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Formation of the transition zone by Mks5/Rpgrip1L establishes a ciliary zone of exclusion (CIZE) that compartmentalises ciliary signalling proteins and controls PIP2 ciliary abundance

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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.)

Editor: David del Álamo

1st Editorial Decision 27 February 2014

Thank you once more for the submission of your manuscript "Formation of ciliary transition zone by Mks5/Rpgrip1L compartmentalizes signaling proteins" to The EMBO Journal and please accept again my apologies for the delay in responding. As already indicated, four referees have accepted the invitation to review your manuscript, and we have so far received reports from three of them, which I copy below. As two of these referees are convinced about the high interest, novelty and quality of your study, I would like to ask you to begin revising your manuscript according to the his/her comments. Please note that this decision is made in the interest of time, and I will forward you the fourth report, probably including further requests, as soon as I receive it.

Without going into all the details that you will find below, referees #1 and #2 are very positive as I already mentioned, although referee #2 is moderately concerned about the lack of mechanistic evidence in some cases as well as certain controls. Referee #3's comments, more negative in general, also point towards the lack of suitable controls for some of your experiments, although s/he agrees on the overall interest of your manuscript for The EMBO Journal. After discussion within our editorial team, we consider that the assays required to deal with these concerns are rather standard and feasible within the timeframe allotted to the revision of your manuscript. That being said, do not hesitate to contact me by e-mail or on the phone if you have any questions, you need further input or you anticipate any problems during the revision process.

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In this regard, please note that it is 'The EMBO Journal' policy to allow a single round of major revision only and that we generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). Nevertheless, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

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

REFEREE COMMENTS

Referee #1:

The transition zone (TZ) at the base of the cilium provides a barrier (ciliary gate) to free diffusion of substances into and out of the cilium. The TZ has attracted a great deal of recent attention because mutations in its components lead to devastating human pathologies. One model system to explore the problem of TZ structure and function is C.elegans, whose sensory neurons are ciliated. In this ms, using mutants and fluorescent imaging of proteins the system is superbly used to produce new information defining the assembly and structure of the TZ and with real imagination to characterize a lipid component PIP2 of the TZ. The authors show that structure and assembly of the two protein modules (MSK and NPHP) of the TZ depend on a master protein MSK-5. After they characterize this extensively, including studies of MSK-5 truncation, they show that mutating MSK-5 changes the residence of signaling proteins, leading to a zone of exclusion (CIZE) within the TZ, and also helps to define the specialized inversin compartment of the cilium. These studies depend on very small distinctions of fluorescent localization within the TZ and along the cilium, which require expert interpretation, but are for the most part convincing, especially when accompanied by interpretative diagrams. They then present several provocative models of how MSK-5 could build the TZ and how compartmentalization of signaling proteins could work. They also discuss the mammalian homologies, which increases the value of the study for the field. The models will probably require later revision, but are very useful summaries of the conclusions for the reader. This is a careful, extensive and very interesting study with no major problems for expedited publication.

Two things to consider:
(1) The "Y links". These are actually 3D structures as originally described. Protein organization must be 3D. Can you comment?
(2) p. 13 To get to the axoneme all proteins must pass through CIZE. Why aren't they found there? Maybe mention and clarify your hypothesis

A few minor comments:
p.8 Fig 7A (not 6A)
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p.12 Fig 4C and Fig legend p.39. This is difficult to follow for a non-C. elegans expert. What do the different stain intensities mean?

Referee #2:

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Title

Formation of ciliary transition zone by Mks5/Rpgrip1L establishes a PIP2-enriched ciliary zone of exclusion (CIZE) needed for compartmentalising signaling proteins

Synopsis

This report describes the role of MKS-5 as a core transition zone component of nematode cilia. Deletion of MKS-5 results in loss of MKS and NPHP module components that result in loss of ciliary membrane attachment to the transition zone. This phenotype explains previous work in which MKS module component loss is synthetic with NPHP module component loss. In addition to loss of membrane attachment, MKS-5 loss also results in the loss of a membrane exclusion zone coincident with the transition zone, causing an extended localization of NPHP-2/Inversin and transmembrane receptors. Moreover, MKS-5 is coincident with a PI(4,5)P2 lipid-rich region, that is partially disrupted in MKS-5 deleted cilia. Together, the data identify MKS-5 as the apical protein in an assembly hierarchy that includes both the MKS and NPHP modules and that the transition zone is responsible for a specific lipid domain and a protein exclusion domain in the transition zone.

General Comments

The manuscript reports a wide variety of observations related to the disruption of MKS-5. While individually they are important demonstrations of specific correlative roles for MKS-5, much of the data does not provide specific mechanistic insight. For example, the increase in size for the Inversin domain or PI(4,5)P2 domains after MKS-5 disruption show that part of the MKS or NPHP modules are important for creating (or maintaining) this region. However, the manuscript provides little analysis of what specific components of these modules are responsible. Without this data it is difficult to ascribe activities that are at the axoneme or at the membrane that control these functions. Because of this, and other over-interpretations of the data, the last figure is particularly troublesome. The mechanisms proposed in the later panels (c,d,e) are intriguing but are not supported by the data in the manuscript.

For panel c: there are a number of configurations that can give the MKS-5 localization and localization hierarchy observed. The role of a scaffold or assembly factor are both possible but no specific data in this manuscript discriminates between them and certainly do not support the two models proposed as the leading hypotheses. For panel d: the evidence for specific lipid domains within the cilium is already widely known. The use of dyes such as DiI must be done carefully since the size of the hydrocarbon chain can often give distinct localizations in cells (e.g. mitochondrion [<12C] vs, plasma membrane [>12C]) and given the unique aspects of the cilium interpretations should be conservative. Here a DiI localization on the mks-5 background could also be helpful. It should be noted that the Plcdelta1 PH domain can also be affected by PE and cholesterol levels (e.g. Flesch et al, 2005 Biochemical Journal). Panel e is widely speculative and provides some testable hypotheses, which are themselves difficult to extract from the data presented here.

One element of the manuscript that does provide some mechanistic understanding is the mutant MKS-5 (in the C2 domain). Learning what proteins in the NPHP and MKS modules are disrupted in this mutant as well as changes in the DiI or PH domain localization would be interesting. A causal mechanism may be difficult to ascertain, but this would begin to dissect domains in MKS-5 that support building the TZ and membrane domains.

Another observation on which the authors spend a good deal of time is MKS-5 doublet seen in the MKS and NPHP mutants. While intriguing, the exact cause of this could be manifold and the authors do not provide any specific staining (i.e. what's between the two MKS-5 dots) or ultrastructure to support a specific mechanism. Use of the new genetic EM tags (e.g. APEX or miniSOG) may a useful method to study this phenomenon.

In summary, the data presented uses a number of genetic models to show an assembly hierarchy down stream of MKS-5, it introduces a number of new TZ components. The function of MKS-5 is addressed by observing changes in the domain of lipids and other ciliary proteins (e.g. Inversin and GPCRs) but given it's master role in assembly of the TZ, a mechanistic understanding is not available at this time. The data is quite compelling, but not enough to support the mechanisms

proposed in the final figure. Moreover, the use of PH domains and lipophilic dyes should be done with more care to be sure that they are reporting what they think. Given the unique properties of the ciliary membrane, it should not be taken for granted that these reagents will act as previously described.

Specific Comments

1. Throughout the manuscript, there are many grammatical errors. The authors should carefully check their prose.

Referee #3:

Cilia are sensory and motile organelles that perform important roles in human physiology and development. This manuscript focuses on the transition zone, a structure at the base of the cilium characterized by Y-links linking axonemal microtubules to the ciliary membrane that controls access to the ciliary compartment. This structure has been the subject of numerous studies in C. elegans and elsewhere examining the assembly and function of various ciliopathy-related proteins.

Central to this manuscript is MKS-5, a protein previously shown to be important for assembly of the transition zone in C. elegans. Based primarily on its localization, the authors propose that MKS-5 functions as an assembly factor, rather than as a scaffold, for transition zone proteins, including three newly identified components. They further describe a "ciliary zone of exclusion" at the transition zone defined by a particular membrane composition which may contribute to ciliary compartmentalization.

As detailed below, this manuscript suffers from serious deficiencies in experimental design, validation of reagents and presentation of data. I therefore consider this manuscript unsuitable for publication in its present form.

Major criticisms

1. Large parts of the manuscript are based on the use of reagents that have not been properly characterized or validated. In particular, mutants of tmem-17, mks-2 and tmem-138 are described as 'likely nulls' (page 6) without any experimental support (RT-PCR, Western blot or immunofluorescence) for such a claim or even a short description of the nature of each mutant.

2. Background mutations introduced during random mutagenesis are also a concern, particularly for mutants obtained from the Million Mutation Project. Outcrossing 4x against wild-type is barely adequate, and based on the description in the Materials and Methods section no outcrossing at all was done for the mks-5 missense mutants. It is also disconcerting that (dyefill) phenotype rather than genotype (presence of the mutation) was used to track missense mutations through crosses, thereby selecting for the very phenotype that is being scored.

3. Given that localization patterns observed are solely based on the use of fluorescent fusions, their functionality needs to be confirmed by rescue of the respective mutant. This would also go some way to addressing point 2, above.

4. With the exception of the electron microscopy data, scalebars are missing for all image panels in the manuscript, making it difficult to interpret what is being presented.

5. No information is presented on the nature of the statistical tests performed in Figs. 3,5, 6 and S1. Asterisks are undefined in Figs. 5B,D,E and S1B, and quantitations in Figs 3C, 5D and 6F lack error bars. The term 'staining intensity' as a measure of dyefilling efficency in Figs 4C and S1A is also undefined. Given that this assay is largely all or none for a particular neuron, what is the difference between +/- in Fig. 4C and - in Fig. S1A?

6. The idea of MKS-5 acting as a molecular chaperone rather than a scaffold for transition zone assembly is interesting, but differential localization alone is not sufficient evidence. For example, CENP-A functions as a scaffold for kinetochore assembly, despite not colocalizing with outer

kinetochore components. It should further be noted that the 2-dot localization pattern observed is based on the use of an mCherry fusion in a wild-type mks-5 background. It is thus possible that endogenous MKS-5 displaces the bulkier fusion protein to the transition zone periphery.

7. Given the emphasis the authors place on the enrichment of PIP2 at the transition zone, the data in support of this in Fig. 6 is underwhelming. Given that PIP2 is a key constitutent of the plasma membrane including in C. elegans, some localization of the PH domain probe at the ciliary membrane is to be expected. It is difficult to discern any enrichment at the transition zone from the images presented (if anything the periciliary compartment below the transition zone displays a more prominent enrichment that is independent of MKS-5). Given that this signal is observed in only two specialized neuronal cell types (the male-specific CEM and ray neurons) despite presumably using a pan-neuronal or pan-cilia promoter (description of the reporter construct is missing from the Materials and Methods section) one cannot talk of PIP2 enrichment as a defining feature of the transition zone.

8. The ultrastructural analysis is one of the stronger points of this manuscript, even if the results are largely unsuprising. One striking anomaly, however, is the strong phenotype (fragmentation of the transition zone) observed in mks-5 mutants. This stands in apparent contrast to the milder phenotype shown previous for mks-5;nphp-4 double mutants (Williams et al., 2011). The authors need to address this discrepancy.

9. Of the three novel (in C. elegans) transition zone components, little data is shown for TMEM-231, apparently since the authors are preparing a separate manuscript on this protein. The authors should either include a more comprehensive analysis, in particular of its place in the transition zone assembly hierarchy, or remove it from the manuscript.

Minor points

10. In the abstract human MKS5 and RPGRIP1L are presented as different proteins even though they are alternative names for the same one, with RPGRIP1L the official HUGO-approved name. This should be corrected.

11. The localization (non-)interdependency map in Fig. S1D is counterintuitive and redundant with the more convential map presented in Fig. 7A and should be removed.

1st Revision - authors' response 06 July 2015

Response to reviewers:

<u>REFEREE #1</u>:

The transition zone (TZ) at the base of the cilium provides a barrier (ciliary gate) to free diffusion of substances into and out of the cilium. The TZ has attracted a great deal of recent attention because mutations in its components lead to devastating human pathologies. One model system to explore the problem of TZ structure and function is C.elegans, whose sensory neurons are ciliated. In this ms, using mutants and fluorescent imaging of proteins the system is superbly used to produce new information defining the assembly and structure of the TZ and with real imagination to characterize a lipid component PIP2 of the TZ. The authors show that structure and assembly of the two protein modules (MSK and NPHP) of the TZ depend on a master protein MSK-5. After they characterize this extensively, including studies of MSK-5 truncation, they show that mutating MSK-5 changes the residence of signaling proteins, leading to a zone of exclusion (CIZE) within the TZ, and also helps to
define the specialized inversin compartment of the cilium. These studies depend on very small distinctions of fluorescent localization within the TZ and along the cilium, which require expert interpretation, but are for the most part convincing, especially when accompanied by interpretative diagrams. They then present several provocative models of how MSK-5 could build the TZ and how

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compartmentalization of signaling proteins could work. They also discuss the mammalian homologies, which increases the value of the study for the field. The models will probably require later revision, but are very useful summaries of the conclusions for the reader. This is a careful, extensive and very interesting study with no major problems for expedited publication.

Two things to consider:
(1) The "Y links". These are actually 3D structures as originally described. Protein organization must be 3D. Can you comment?

Yes, Y-links are known to be large electron-dense structures that presumably have multiple different proteins forming the structure. In this study we analyse several of these proteins, many of which are associated with the membrane (either transmembrane or peripherally associated with membrane). We also investigate MKS-5, which we suggest might act as a scaffolding protein for the assembly of the transition zone, including the Y-links.

(2) p. 13 To get to the axoneme all proteins must pass through CIZE. Why aren't they found there? Maybe mention and clarify your hypothesis

We have expanded on our discussion of the nature of the CIZE, in that it is made up of many membrane proteins, as well as a specific lipid content that would serve to exclude membrane proteins.

A few minor comments:
p.8 Fig 7A (not 6A)

Fixed.

p.9 Fig1E Two locations not clear. What do the enlargements show?

As stated in the manuscript, the two localizations of NPHP-1 and NPHP-4 are adjacent (basal body and transition zone) to each other and therefore appear as one longer contiguous signal.

p.10 Two foci of MSK-5. This is only found in mutants, not wt -but this is the basis for the major model. Is this a worry? Add a sentence of explanation?

We appreciate that the reviewer would require additional explanation for the basis of one of our models. We have now expanded the section where we describe that these foci are too close to be distinguished by confocal microscopy. Immunogold staining in retinal transition zones (connecting cilium) support the notion that Rpgrip1 (orthologous to MKS-5) can be observed at the two flanking ends of the transition zone. This means that Rpgrip1/MKS-5 would not be part of the final assembled Y-links themselves. At the same time, we suggest that additional experiments, including superresolution microcospy, will be useful to address this question directly in *C. elegans*.

p.12 Fig 4C and Fig legend p.39. This is difficult to follow for a non-C. elegans expert. What do the different stain intensities mean?

The loss of staining intensity is indicative of ciliary structural defects; we have clarified this in text.

REFEREE #2:

Title

Formation of ciliary transition zone by Mks5/Rpgrip1L establishes a PIP2-enriched ciliary zone of exclusion (CIZE) needed for compartmentalising signaling proteins

Synopsis

This report describes the role of MKS-5 as a core transition zone component of nematode cilia. Deletion of MKS-5 results in loss of MKS and NPHP module components that result in loss of ciliary membrane attachment to the transition zone. This phenotype explains previous work in which MKS module component loss is synthetic with NPHP module component loss. In addition to loss of membrane attachment, MKS-5 loss also results in the loss of a membrane exclusion zone coincident with the transition zone, causing an extended localization of NPHP-2/Inversin and transmembrane receptors. Moreover, MKS-5 is coincident with a PI(4,5)P2 lipid-rich region, that is partially disrupted in MKS-5 deleted cilia. Together, the data identify MKS-5 as the apical protein in an assembly hierarchy that includes both the MKS and NPHP modules and that the transition zone is responsible for a specific lipid domain and a protein exclusion domain in the transition zone.

General Comments

The manuscript reports a wide variety of observations related to the disruption of MKS-5. While individually they are important demonstrations of specific correlative roles for MKS-5, much of the data does not provide specific mechanistic insight. For example, the increase in size for the Inversin domain or PI(4,5)P2 domains after MKS-5 disruption show that part of the MKS or NPHP modules are important for creating (or maintaining) this region. However, the manuscript provides little analysis of what specific components of these modules are responsible. Without this data it is difficult to ascribe activities that are at the axoneme or at the membrane that control these functions.

> We thank the referee for their constructive comments. We have found that MKS-5 is critical for compartmentalising signalling proteins like inversin to the distal end of the ciliary axoneme. We have indeed tested which other proteins might confer this functionality, which we call ciliary zone of exclusion (CIZE). We discovered that other transition zone mutants, as well as double mutants, do not have an effect on this CIZE. Our data is therefore consistent with MKS-5 playing a potential central, and perhaps direct, role in forming the CIZE.

Because of this, and other over-interpretations of the data, the last figure is particularly troublesome. The mechanisms proposed in the later panels (c,d,e) are intriguing but are not supported by the data in the manuscript.

For panel c: there are a number of configurations that can give the MKS-5 localization and localization hierarchy observed. The role of a scaffold or assembly factor are both possible but no specific data in this manuscript discriminates between them and certainly do not support the two models proposed as the leading hypotheses.

> We appreciate the fact that the two potential models for transition zone assembly by MKS-5/Rpgrip1L, namely as a scaffold for proteins that for Y-links, or as an ‘assembly factor’, will require more work. This is the first time, to our knowledge, that the ‘assembly factor’ model is proposed, and as noted above is consistent with the finding in mammalian cells that Rpgrip1 appears to flank the transition zone Y-link-containing structures. We propose that superresolution microscopy—which has not yet been used in *C. elegans*, probably owing to technical difficulties of visualising structures deep within the animal (we have tried)—will be helpful to visualise MKS-5 flanking other transition zone proteins (e.g., MKS-2) in a wild-type situation. This would again provide evidence complementing ours that MKS-5 does not specifically co-localise with the Y-links and their associated proteins. At this moment, such studies would require advances in microscopy techniques applicable to C. elegans that we deem to be beyond the scope of this study.

For panel d: the evidence for specific lipid domains within the cilium is already widely known. The use of dyes such as DiI must be done carefully since the size of the hydrocarbon chain can often give distinct localizations in cells (e.g. mitochondrion [<12C] vs, plasma membrane [>12C]) and given the unique aspects of the cilium interpretations should be conservative. Here a DiI localization on

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the mks-5 background could also be helpful. It should be noted that the Plcdelta1 PH domain can also be affected by PE and cholesterol levels (e.g. Flesch et al, 2005 Biochemical Journal).

We agree with the referee that different lipid markers could give distinct localisations in cells, and this was the reason to test for DiI staining. We wondered if it might be excluded from the transition zone, but it is not and so was not an appropriate marker to use to test transition zone gate function. We saw no difference for DiI in wild type and *mks-5* and thus have not included it because it was not informative. We do think however that our result is informative that a freely diffusing dye is excluded from a membrane compartment, given that lipidated proteins that freely diffuse in the ciliary middle segment membrane are also excluded, indicating that there is some form of barrier at the middle segment/distal segment interface. Indeed, the PLCdelta PH domain can be affected by PE and cholesterol, and this might be the reason for a differential distribution between the periciliary membrane and the cilium (new result we show). We also note that the affinity for PIP2 is well-studied and established, and given that PIP2 has been connected to cilium biology and Joubert disease specifically (through INPP5E and ARL13B), our data are consistent with the disease phenotype, and are similar to those obtained in Trypanosomes (PIP2 at the base of, but not within, cilia; we have added this reference in the manuscript).

Panel e is widely speculative and provides some testable hypotheses, which are themselves difficult to extract from the data presented here.

Given the data published by the Jackson lab indicating that Tubby requires PIP2 and IFT to deliver GPCRs to cilia, and that worm tubby homologue (tub-1) is also required for localisation of GPCRs to the cilium, we have proposed a model for PIP2 function at the ciliary base. In this model, the concentrations of PIP2 would be high at the base of cilia (we demonstrate this and it is true in Trypanosomes), and lower within cilia (we show this and it is true in Trypanosomes) since GPCRs would need to be released. Another study by the Maureen Barr lab, which we cite also demonstrates that a PIP2 phosphatase (CIL-1) is required for trafficking of a ciliary protein, consistent with our model. We have made it clear that our data is a model based on our work and other published studies, and a testable model as the referee noted, so we feel that as part of the discussion we can propose this.

One element of the manuscript that does provide some mechanistic understanding is the mutant MKS-5 (in the C2 domain). Learning what proteins in the NPHP and MKS modules are disrupted in this mutant as well as changes in the DiI or PH domain localization would be interesting. A causal mechanism may be difficult to ascertain, but this would begin to dissect domains in MKS-5 that support building the TZ and membrane domains.

We agree with the referee that we have begun to dissect the function of MKS-5 but that more could be done to ascertain how the different parts of the protein might influence DiI or PH domain (PIP2) localisation/distribution. However, we feel that such a study would be highly involved, and would for example require ascertaining the exact composition of transition zone proteins in the various MKS-5 truncation mutant strains, coupled with TEM analyses to understand how these influence the structure of the transition zone. As such, such experiments are beyond the scope of this work.

Another observation on which the authors spend a good deal of time is MKS-5 doublet seen in the MKS and NPHP mutants. While intriguing, the exact cause of this could be manifold and the authors do not provide any specific staining (i.e. what's between the two MKS-5 dots) or ultrastructure to support a specific mechanism. Use of the new genetic EM tags (e.g. APEX or miniSOG) may a useful method to study this phenomenon.

We agree that the exact nature of the doublets we observe with MKS-5 remains unclear at this moment. We have attempted to use superresolution microscopy to be able to observe these, together with other co-markers (as the referee suggests) to see MKS-5 signals flanking other transition zone protein signals. We have not had luck thus far, and note that there are difficult-to-surmount obstacles to carrying out supreresolution microscopy in *C. elegans*. Furthermore, a collaborator has been working on miniSOG, but with little success. We have nevertheless quantitated our confocal microscopy observations, and found that

doublets are only observed with MKS-5, supporting the notion that it flanks other transition zone proteins.

In summary, the data presented uses a number of genetic models to show an assembly hierarchy down stream of MKS-5, it introduces a number of new TZ components. The function of MKS-5 is addressed by observing changes in the domain of lipids and other ciliary proteins (e.g. Inversin and GPCRs) but given it's master role in assembly of the TZ, a mechanistic understanding is not available at this time. The data is quite compelling, but not enough to support the mechanisms proposed in the final figure. Moreover, the use of PH domains and lipophilic dyes should be done with more care to be sure that they are reporting what they think. Given the unique properties of the ciliary membrane, it should not be taken for granted that these reagents will act as previously described.

We appreciate that care must be taken to interpret our results. There is compelling evidence that the PH domain we used specifically binds PIP2, which would support our model that it is present at the base of cilia, and that its abundance in the cilium is at least in part controlled by MKS-5 and the transition zone. Our data are the first to be presented on the localisation of PIP2 and the effect of a transition zone protein, and are consistent with the notion that PIP2 is central to ciliopathies such as Joubert syndrome. As such, we feel that the work provides a strong hypothesis for the function of the MKS-5 and the transition zone and merit further studies to expand and refine the model.

Specific Comments

1. Throughout the manuscript, there are many grammatical errors. The authors should carefully check their prose.

We thank the referee for pointing this out. We have endeavoured to review and revise the manuscript to fix any lingering errors.

REFEREE #3:

Cilia are sensory and motile organelles that perform important roles in human physiology and development. This manuscript focuses on the transition zone, a structure at the base of the cilium characterized by Y-links linking axonemal microtubules to the ciliary membrane that controls access to the ciliary compartment. This structure has been the subject of numerous studies in C. elegans and elsewhere examining the assembly and function of various ciliopathy-related proteins.

Central to this manuscript is MKS-5, a protein previously shown to be important for assembly of the transition zone in C. elegans. Based primarily on its localization, the authors propose that MKS-5 functions as an assembly factor, rather than as a scaffold, for transition zone proteins, including three newly identified components. They further describe a "ciliary zone of exclusion" at the transition zone defined by a particular membrane composition which may contribute to ciliary compartmentalization.

As detailed below, this manuscript suffers from serious deficiencies in experimental design, validation of reagents and presentation of data. I therefore consider this manuscript unsuitable for publication in its present form.

Major criticisms

1. Large parts of the manuscript are based on the use of reagents that have not been properly characterized or validated. In particular, mutants of tmem-17, mks-2 and tmem-138 are described as 'likely nulls' (page 6) without any experimental support (RT-PCR, Western blot or immunofluorescence) for such a claim or even a short description of the nature of each mutant.

We have added descriptions of these mutations.

2. Background mutations introduced during random mutagenesis are also a concern, particularly for mutants obtained from the Million Mutation Project. Outcrossing 4x against wild-type is barely adequate, and based on the description in the Materials and Methods section no outcrossing at all was done for the mks-5 missense mutants. It is also disconcerting that (dyefill) phenotype rather than genotype (presence of the mutation) was used to track missense mutations through crosses, thereby selecting for the very phenotype that is being scored.

We agree that this was a shortcoming for this experiments; it was done to screen an allelic series, and we have now shown rescue of the phenotype of the MMP allele.

3. Given that localization patterns observed are solely based on the use of fluorescent fusions, their functionality needs to be confirmed by rescue of the respective mutant. This would also go some way to addressing point 2, above.

We have now shown rescue for the TMEM-17 (dye-filling of double mutant), MKS-2 (dye-filling of double mutant) and MKS-5 (localization of NPHP-1 and MKS-2, as well as rescue of the mks-5(gk153561);nphp-4 dye-filling) constructs.

4. With the exception of the electron microscopy data, scalebars are missing for all image panels in the manuscript, making it difficult to interpret what is being presented.

We have added scale bars.

5. No information is presented on the nature of the statistical tests performed in Figs. 3,5, 6 and S1. Asterisks are undefined in Figs. 5B,D,E and S1B, and quantitations in Figs 3C, 5D and 6F lack error bars. The term 'staining intensity' as a measure of dyefilling efficency in Figs 4C and S1A is also undefined. Given that this assay is largely all or none for a particular neuron, what is the difference between +/- in Fig. 4C and - in Fig. S1A?

We have added more information about the statistical analyses, and the dye-filling phenotype is has been previously shown to not be all or none. The + or +++ or +/- is based on a reproducible, qualitative analysis.

6. The idea of MKS-5 acting as a molecular chaperone rather than a scaffold for transition zone assembly is interesting, but differential localization alone is not sufficient evidence. For example, CENP-A functions as a scaffold for kinetochore assembly, despite not colocalizing with outer kinetochore components. It should further be noted that the 2-dot localization pattern observed is based on the use of an mCherry fusion in a wild-type mks-5 background. It is thus possible that endogenous MKS-5 displaces the bulkier fusion protein to the transition zone periphery.

The referee is correct that colocalisation alone is not sufficient to ascribe functional relationships. If, as stated above, we had clear-cut colocalisation of MKS-5 and different transition zone proteins by superresolution microscopy, our data would be even more clear. Having said that, the published immunogold staining for RPGRIP1 in photoreceptors is dramatic (the transition is very long and RPGRIP1 is only at the flanking ends), and we have quantitated the 2-dot localisations we observe in our experiments, analyzing these in two different (*mks-2* and *mks-3*) mutants. We are therefore confident that the *localisation behaviour* of MKS-5 is different from that of the other transition zone proteins, suggesting that they are not part of the same assembly. It should also be noted that we present this as a potential model, together with the possibility that MKS-5 could potentially ‘simply’ act as a scaffold.

7. Given the emphasis the authors place on the enrichment of PIP2 at the transition zone, the data in support of this in Fig. 6 is underwhelming. Given that PIP2 is a key constitutent of the plasma membrane including in C. elegans, some localization of the PH domain probe at the ciliary membrane is to be expected. It is difficult to discern any enrichment at the transition zone from the images presented (if anything the periciliary compartment below the transition zone displays a more prominent enrichment that is independent of MKS-5). Given that this signal is observed in only two

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specialized neuronal cell types (the male-specific CEM and ray neurons) despite presumably using a pan-neuronal or pan-cilia promoter (description of the reporter construct is missing from the Materials and Methods section) one cannot talk of PIP2 enrichment as a defining feature of the transition zone.

We would like to thank the referee for questioning our results, because we have now spent a considerable amount of time and effort to accurately assess the localisation of the PH domain (PIP2) and the effect of disrupting MKS-5. To visualise the marker in additional ciliated neurons, we have now put the PLCdeltaPH::GFP construct under the pan-ciliated promoter *bbs-8*. We have also co-marked the male-specific construct with the IFT protein XBX-1 and the pan-ciliated neurons with MKSR-2 (transition zone protein), as well as TRAM-1 (periciliary membrane marker). Using these many different new strains, we confirm by careful quantitation of the PIP2 distribution that it is present within the transition zone (Fig. 6). However, we have now revised where the bulk distribution of PIP2 is, namely, within the periciliary membrane (*i.e.*, at the base of cilia). Notably, we note that this is the same as that found at the base of Trypanosome cilia. Furthermore, we find that disruption of MKS-5 (transition zone) affects the distribution of PIP2, so that a much higher concentration is found within the cilium. We have revised our models showing how the transition zone 'gates' PIP2 (Fig. 7D) and how PIP2 could be used to facilitate the trafficking of GPCRs across the transition zone (Fig. 7E).

8. The ultrastructural analysis is one of the stronger points of this manuscript, even if the results are largely unsuprising. One striking anomaly, however, is the strong phenotype (fragmentation of the transition zone) observed in mks-5 mutants. This stands in apparent contrast to the milder phenotype shown previous for mks-5;nphp-4 double mutants (Williams et al., 2011). The authors need to address this discrepancy.

We do indeed see some fragmentation in our Willams et al. 2011 study (Fig. S2, panel B), consistent with our observations herein. We now include a reference to that specific TEM in the manuscript.

9. Of the three novel (in C. elegans) transition zone components, little data is shown for TMEM-231, apparently since the authors are preparing a separate manuscript on this protein. The authors should either include a more comprehensive analysis, in particular of its place in the transition zone assembly hierarchy, or remove it from the manuscript.

We agree with the referee that we were not showing much data on TMEM-231, given that we were preparing a much more thorough focussed on this protein. We have now removed the data from the manuscript.

Minor points

10. In the abstract human MKS5 and RPGRIP1L are presented as different proteins even though they are alternative names for the same one, with RPGRIP1L the official HUGO-approved name. This should be corrected.

We have now fixed this.

11. The localization (non-)interdependency map in Fig. S1D is counterintuitive and redundant with the more convential map presented in Fig. 7A and should be removed.

We appreciate that the non-interdependency map we present is counterintuitive, since normally, we only present 'positive' data. Nevertheless, knowing that an experiment has been done to localise a protein in a particular mutant, and that there was no mislocalisation, actually provides additional data. For this reason, we have decided to retain this (also, it is presented as a supplementary figure, so it does not take up space in the main manuscript).

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2nd Editorial Decision 14 August 2015

Thank you for the submission of your revised manuscript and please accept my apologies for the delay in responding. As you will see below, your article was sent back to the referees, who with the exception of referee #3, now consider that you have properly dealt with the main concerns originally raised. I am therefore writing with an 'accept in principle' decision. This means that I will be happy to formally accept your manuscript for publication once a few more minor issues are dealt with.

All referees essentially agree that due to the extreme complexity of the system, and despite the enormous wealth of data presented, the picture of MKS5 function is still incomplete. However, while referee #1 and #2 consider that future papers should address this issue, referee #3 is more negative and does not recommend publication in The EMBO Journal. After discussing this issue with the referees as well as other members of the editorial team, we are now convinced that in the absence of any serious technical or conceptual flaws, the publication of the paper should not be prevented by differences in the interpretation of the data, or even the lack of absolute completeness of the conclusions presented. We have therefore decided to publish your (very timely) manuscript and let the community decide.

As mentioned above, only very minor potential corrections and clarifications might be needed as indicated by referee #1. In addition, as you might know, every article now includes a 'Synopsis' to further enhance their discoverability. Synopses are displayed on the html version of the article and they are freely accessible to all readers. The synopsis includes an image, as well as 2-5 one-short-sentence bullet points that summarize the article. I would be grateful if you could provide both the figure (a slightly modified version of Fig. 7A will do) and the bullet points for your article.

If you have any questions or need any further input, please do not hesitate to contact me.

Thank you very much for your patience and congratulations in advance for a successful publication!

Referee #1:

This revised version of this important paper addresses the issues I previously raised and responds to the concerns of the other reviewers. I do not work with C.elegans and cannot address the genetic methodology adequately but from my perspective with very minor corrections the ms should now be published without delay. There is great general current interest in the structure of the ciliary transition zone, which this ms addresses with new insights and elegant analysis of immunofluorecent light and electron micrographs, accompanied by interpretive diagrams and functional hypotheses. While I agree that superresolution microscopy and new Immuno EM would resolve some of the issues raised, there is enough information to be digested in this paper and so many subtle points raised and discussed that these techniques are really for the next phase of the work. The concept of a transition zone being a ciliary zone of exclusion-meaning that signaling and other molecules pass through but do not remain concentrated in this region while entering (and leaving?) the cilium is useful. The idea that this is partly dependent on PIP2 is interesting and will provide a basis for further study, especially of the kinetics of transport. The Information on IFT transport through the TZ is a good start. There is still a lot to be tested but the information provided that leads to these ideas is solid,appropriately illustrated, qualified and available to the reader.

Two suggestions:

Please go over the text citations to various figures again carefully. I found several misstatements: on p.12 aa mutations shown in Fig 4a , not 4b; p.15 MSKR2 construct not MSKR1 (no MSKR1 in Fig 6c; p.16 CIZE in Fig 7a, not 7b. The figures are complex ad misdirection confuses the reader.

The ms still has problems with is the dual appearance of MSK5 in the TZ mutants. The penultimate paragraph of the ms attempts an explanation but perhaps pushes a bit too hard since as the authors say this is a question that awaits further resolution. This paragraph could be revised and shortened to make this point more succinctly.

Referee #2:

Excellent revision. While the detailed mechanisms are still incomplete, the new data provides a wealth of new phenomena that are regulated by the TZ. This work represents an important step in building a mechanistic model of TZ function.

Referee #3:

In my original comments on the manuscript by Jensen and colleagues submitted more than a year ago I highlighted a number of serious deficiencies in experimental design, validation of reagents and presentation of data that for me precluded further consideration of this work. This revised manuscript goes some way towards remedying those shortcomings. However, given that the data presented fails to support the expansive conclusions made by the authors I remain unconvinced as to its suitability for publication in The EMBO Journal.

On a positive note, the manuscript now includes scalebars in image panels and error bars on graphs, and statistical tests are defined in the text. The Materials and Methods section also now includes short descriptions of novel mutant alleles, although there is still no experimental validation to support the claim that these (or indeed the original mks-5(tm3100) mutant) are truly null alleles. The authors do, however, demonstrate rescue of mks-2, mks-5 and tmem-17 double mutants with nphp-4 by expression of the respective GFP transgene, simultaneously validating both mutant and GFP fusion.

Where the manuscript continues to fall short is in presenting a coherent picture of MKS-5 that provides new mechanistic insight into its role at the transition zone. That MKS-5 is important for proper localization of MKS and NPHP module components had already been demonstrated in the authors' previous study (Williams et al, JCB 2011). As also commented on by referee #2, the 2-dot localization of MKS-5 in various transition zone mutants and perhaps also the wild-type is curious but does not distinguish between the two models the authors present for MKS-5 function, as a scaffold or chaperone for transition zone assembly.

The other major conclusion of this manuscript is that MKS-5 helps create a ciliary zone of exclusion, in part by regulating the abundance of PIP2 within the ciliary membrane. In my previous comments I raised doubts on the enrichment the authors claimed to detect for PIP2 within the transition zone (a claim repeated in the title, no less). The authors now report that PIP2 is actually excluded from the transition zone based on quantitation of GFP:PH domain signal in a different set of neurons (phasmids). This radical change in interpretation does not inspire much confidence in the authors' conclusions, nor is it adequately explained why the new set of pictures for male tail ray neurons (Fig 6B, current submission), showing exclusion of PIP2 from the cilium in the wild-type, is any more representative than the previous set (Fig 6D, 1st submission), which appeared to show the opposite.

While there is a wide range of data presented in the manuscript (ultrastructural characterization of tmem-17, mks-2 and mks-5 mutants, integration of TMEM-17 and MKS-2 into established transition zone assembly hierarchies, structure-function analysis of MKS-5), there is no coherent message emerging, nor much in the way of novel mechanistic insight. For example, does MKS-5 directly control PIP2 gating or are downstream factors in the MKS/NPHP modules also involved? Could this perhaps simply be a reflection of transition zone disorganization in mks-5 mutants? The authors' answer to referee #2 here (pointing to the apparent spread of NPHP-2 signal specifically in the mks-5 mutant - there is still a gap) does not adequately address this important point.

2nd Revision - authors' response 23 August 2015

Thank you for giving us the opportunity to submit a final, revised version of our manuscript. Based on referee feedback and your request, we have made the following improvements:

1. The incorrect figure citations within the text, pointed out by reviewer 1, have been corrected.

2. The penultimate paragraph was shortened significantly as requested by reviewer 1.

3. We also made very minor tweaks to two of the figures, to remain consistent throughout; no data was altered, just presentation. N2 was changed to wt (wild-type) in figure 6, and the labels were centered (where needed). In figure 7, panel A, the 2 in PIP2 was made subscript, panel D, PI(4,5)P2 was changed to PIP2. Some of the published negative interactions were missing from figure S1d, these have been added. We have adjusted the image cropping on figure S1a to improve it.

4. We have added the Synopsis text to the manuscript file and have included the figure in the submission. It is a combination of various parts of figure 7 and represents much of the data within the paper.

5. We tweaked a couple of words in the summary to make it clear that PIP2 is not excluded from the cilium, but rather, its abundance is limited.

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