EMBO reports

Myosin VI regulates ciliogenesis by promoting the turnover of the centrosomal/satellite protein OFD1

Elisa Magistrati, Giorgia Maestrini, Carlos Niño, Mariana Lince-Faria, Galina Beznoussenko, Alexandre Mironov, Elena Maspero, Monica Bettencourt-Dias, and Simona Polo
DOI: 10.15252/embr.202154160

Corresponding author(s): Simona Polo (simona.polo@ifom.eu) , Elisa Magistrati (elisa.magistrati@ifom.eu)

Review Timeline:		
	Transfer from Review Commons:	15th Oct 21
	Editorial Decision:	19th Nov 21
	Revision Received:	1st Dec 21
	Accepted:	8th Dec 21

Editor: Deniz Senyilmaz Tiebe

Transaction Report: This manuscript was transferred to EMBO Reports following peer review at Review Commons.

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Manuscript number: RC-2021-00901
Corresponding author(s): Simona, Polo

1. General Statements [optional]

In the present manuscript, we reveal and characterise an unexpected and pivotal role for the unconventional myosin VI motor protein in the regulation of centriole biology and ciliogenesis.

Research highlights are as follows.

- We found that myosin VI interacts with OFD1, a centriolar protein involved in primary ciliogenesis and associated with oral-facial-digital syndrome type 1.
- We found that lack of myosin causes an abnormal recruitment of ODF1 to the centriolar walls and, as a consequence, an accumulation of the distal appendage protein Cep164.
- We provided evidence that the aberrant localization of ODF1 is due to a reduction in the OFD1 fraction that freely exchanges between the centriole and the cytoplasm.
- We found that myosin VI loss triggers a severe defect in ciliogenesis that we propose could be due to an impairment in the autophagic removal of OFD1 from satellites.

We have now performed a full revision, following the reviewers' suggestions.
The revised manuscript contains additional panels/modifications to the existing figures.
We are very grateful to the three reviewers for the critical reading of our manuscript, their overall positive comments on the significance of our study and the numerous useful recommendations that we have implemented to improve our study.

2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

Reviewer #1
(Evidence, reproducibility and clarity):
The manuscript by Magistrati et al. addresses the role of a novel interaction of unconventional myosin VI (MVI) and OFD1, a centriolar protein involved in primary ciliogenesis and associated with oral-facial-digital syndrome type 1. The authors mapped the interaction sites between both proteins and found that MVI regulates OFD1 localization, and its loss is associated with a severe defect in ciliogenesis. They propose that this could be a consequence of aberrations in autophagic removal of OFD1 from the pericentriolar structures, termed as satellites.

The experiments were appropriately designed and I do not have major comments related with that aspects of the work. The broad of methods were appropriately chosen and described. My critique concerns the way of data presentation and discussion.
R. We thank Reviewer #1 for his/her positive comment.

Here is the list of remarks that I would like the authors to take into consideration before making a final decision:
1. Figure 1D, please mark the structures described in the text; the same should be done in Suppl. Figure 3A. Also, the EM images in Figure 1 should be enlarged.
R. We apologize for the missing information. We have modified the figures accordingly.

2. In the last sentence of the first section of the Results the authors use a term "bad behaviour" of a construct. What does it mean?
R. Unfortunately, shorter constructs of the coiled-coils region of OFD1 were barely soluble and this precluded further structural studies. We have revised the text to better explain this concept.

3. In the beginning of the third section of the Results (page 7), the authors state that the they unexpectedly observed impairment in proliferation in MVI depleted cells. Well, such defect was earlier observed by many, including Majewski et. al (2011) J Muscle Res Cell Motil in neurosecretory PC12 cells.
R. We agree with the Reviewer that impairment in proliferation observed in myosin VI-depleted cells was previously reported, but to our knowledge, no studies have clearly linked this phenomenon to the p53 status. We have now added additional proliferation data to better characterize the differences between p53 WT (like PC12, RPE and BJ cells) and p53 null cells (HeLa and Caco-2 cells, new Supplementary Figure 5D-F). We also reported the cited study in the legend for the sake of completeness.

4. I do not understand why the authors do not mention in the discussion the work on interaction MVI with CLIP proteins that are considered as a link between the actin and microtubule systems.
R. We are not sure to have understood which proteins the Reviewer is referring to. Clip 170 was identified as a myosin VI binding partner in Drosophila (Lantz and Miller, 1998); and CLIP-170 and CLIP-190 display atypical patch localization together with myosin VI in Drosophila and mouse neurons (Beaven, 2015), but this seems to be limited to neurons as, in mammalian cells other groups failed to confirm the interaction (Arden 2007). We are sorry if we missed critical papers and are ready to better specify a functional link, in case it might exist.

5. In a legend to Suppl. Figure 3 - I think it should be transmission not transmitted EM.
R. We thank the Reviewer for pointing out this issue. We have now edited the text.

(Significance):
This is a very elegant work providing new insight in myosin VI function as well as etiopathology of the OFD1-related syndrome.
R. We thank Reviewer #1 for his/her positive comments on our manuscript.

Reviewer #2
(Evidence, reproducibility and clarity):

The manuscript by Elisa Magistrati and colleagues characterizes the interaction between myosin VI, a unique motor protein transporting cargos towards the minus end of F-actin, and OFD1 a centriolar protein with key functions in regulating ciliogenesis and autophagy. In light of such interaction, the authors characterize the phenotype of myosin VI knock-down, reporting:

1. *quantitative increase in the recruitment of OFD1 to the centrioles (by immunofluorescence); abnormal recruitment of ODF1 to centriolar walls (by super-resolution SIM microscopy); a reduction in the OFD1 fraction that freely exchanges between the centriole and the cytoplasm (by FRAP);*
2. *increase in the recruitment of CEP164 to parent centrioles;*
3. *reduced capability of the cells to autophagically downregulate the overall abundance of OFD1 and particularly the pool localized at centriolar satellites (by immunofluorescence and immunoblotting);*
4. *impaired ciliogenesis (by immunofluorescence);*
5. *a kind p53-dependent cell cycle arrest that appears independent form the centrosomal defects (by growth analysis, flow cytometry and immunoblotting)*

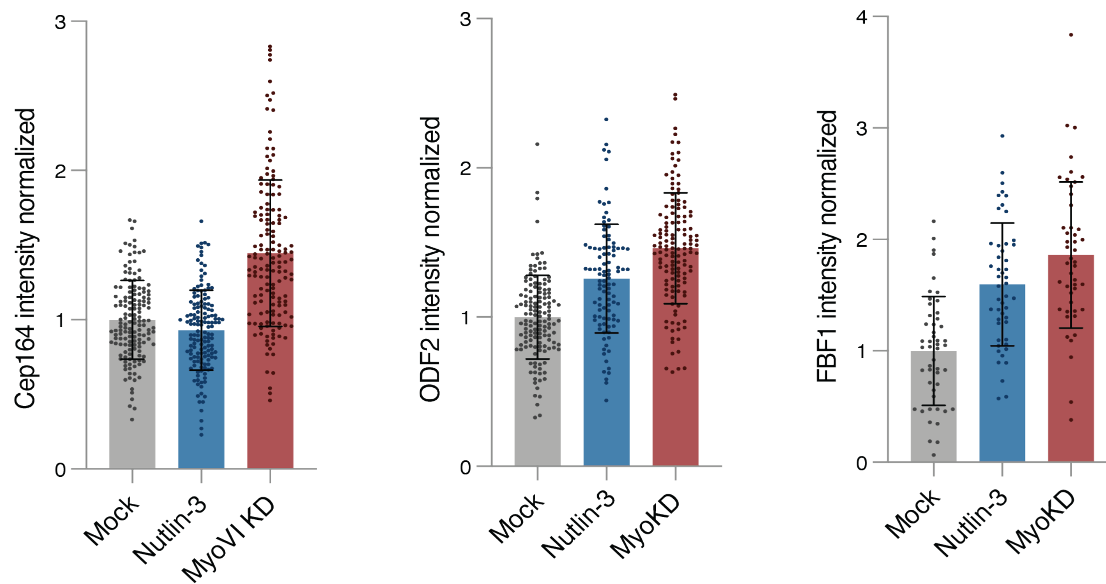
R. We are very grateful to Reviewer #2 for the critical reading of our manuscript and the numerous useful suggestions that we have now implemented to improve our study.

Major comments:

The key conclusions of the manuscript are convincing and well supported by the data, with the following exceptions

1. The increase of CEP164 signal at parent centrioles observed upon myosin VI knockdown is interpreted as a generally increased "distal appendage recruitment" or "increased amount of distal appendages". In normal condition each parent centriole recruits 9 discrete distal appendages and the observed increased recruitment of CEP164 at parent centrioles described here could be consistent with: increase number of distal appendages, distal appendage hypertrophy (along the entire centriole length as for OFD1?) or unbalanced distal appendage protein stoichiometry. As a minimum, the authors could show whether the same pattern is observed for another distal appendage protein (assessing whether this is a general phenomenon) or, as a more extended revision, show super resolution microscopy data to resolve individual distal appendages.

R. We interpreted our Cep164 phenotype as increased distal appendage recruitment, but we totally agree with the Reviewer about the possible alternative explanations. We decided to tackle this issue energetically and perform the requested experiments, analyzing the behavior of the distal appendage protein FBF1 and the subdistal appendage protein ODF2 using SIM and dSTORM. We found that, differently from OFD1, Cep164 and ODF2 localized correctly at the distal end without diffusing along the entire centriole length (new Figure 4B). By dSTORM super resolution microscopy, we could prove that the ninefold symmetrical structures on the distal side of the centriole was not altered in the absence of myosin VI, using both ODF2 and FBF1 as markers (new Figure 4C). On the other hand, both FBF1 and ODF2 intensity levels increase as we observed for cep164, as shown here below.



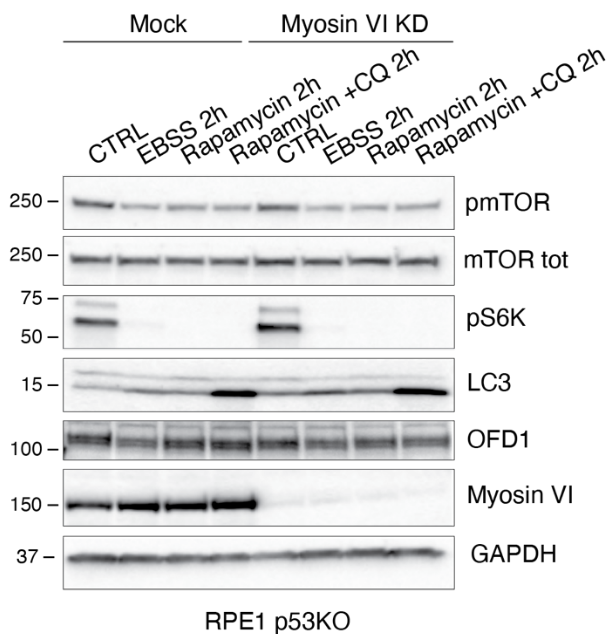
Contrary to Cep164, the intensity levels of ODF2 and FBF1 appear to be sensitive to the Nutlin-3 treatment. Thus, we cannot exclude a contribution of p53 in the phenotype observed upon myosin VI-depletion. Different behavior of these proteins is not surprising, considering the complexity of their assembly. Indeed, ODF2 protein associated with the subdistal appendages, is recruited normally also in OFD1 mutant cells, whereas distal appendage-specific proteins, such as Cep164, are not (Singla et al 2010, DOI 10.1016/j.devcel.2009.12.022). Based on these data, we decided to limit our conclusions to OFD1 and Cep164 for which a more complete analysis was performed and rephrase the corresponding claims.

2. Based on the data presented it is not clear whether the myosin VI knockdown impairs the OFD1 pool at satellites as a result of their direct interaction or of a more general impairment of autophagy. The same is true for ciliogenesis. I think that the overall message of the manuscript should not be affected even when rephrasing the claims associated with the abovementioned points. Thus, it should be up to the authors to either perform the experiments requested for point 1 (might require ca a month and resources for about 1000 €) or address the above-mentioned point by textual edits.

R. As stated in the previous and the following answer, we performed a few experiments to address the points raised by this Reviewer and we hope that the results may satisfy his/her concerns. Nonetheless, we carefully revised the manuscript to better describe our results, avoiding overstatements.

As for point 2, the ciliogenesis defect could depend on
a) Altered OFD1 behavior at the centriole and its impact on distal appendages
b) Defective OFD1 depletion at the centriolar satellites
c) A general effect on autophagy

In light of such consideration, "loss of myosin VI triggers a severe defect in ciliogenesis that could be causally linked to an impairment in the autophagic removal of OFD1 from satellites" in the abstract should be rephrased.
Any step (e.g. the re-expression of a myosin VI mutant capable of supporting autophagy, yet defective in binding to OFD1) that the authors could undertake to better dissect the interdependencies between the many reported phenotypes would improve the mechanistic understanding of the processes and thereby boost the impact of their manuscript. If not readily available, generating such data might prove however more demanding in terms of time and resources. This reviewer does not consider such effort necessary for publishing the work.
R. We agree and thank the Reviewer for his/her anticipated answer that we fully support. While myosin VI was already involved in mitophagy, no data has shown its involvement in this particular form of autophagy called doryphagy (Holdgaard et al 2019). We decided to perform a simple experiment to evaluate a possible general impact of myosin VI on EBSS-induced autophagy but we failed to see differences between WT and myosin VI KD cells. The results are reported below for the Reviewer's eyes. Thus, we could exclude that the ciliogenesis defect depends on a general effect of myosin VI on autophagy.



From this experiment and the new results obtained analyzing other distal appendage proteins, we can fairly conclude that 'impairment in the autophagic removal of OFD1 from satellites' remains the most plausible explanation for the ciliogenesis defect observed. We also modified the sentence in the following way:
" Finally, loss of myosin VI triggers a severe defect in ciliogenesis that could be at least partially ascribed to an impairment in the autophagic removal of OFD1 from satellites. "

All remaining claims (with the exception of the minor comments reported below) of the paper are adequately supported, both in terms of quality of the experimental data generated as well as in terms of statistical robustness.
R. We thank the Reviewer for his/her kind words.

Minor comments:
1. Myosin VI localization at the centrosome shown in Figure S1A. While siRNA mediated depletion of myosin VI appears robust in triggering a number of phenotypes throughout the manuscript, it fails in affecting the centriolar myosin VI antibody staining. Provided that the authors generate and employ A549 derivatives KO for myosin VI in the manuscript, they should address potential antibody cross reactivity issues using this system.

R. We thank the Reviewer pointing out this issue. We have now repeated the IF experiment in A549 KO cells with an improved protocol and we could show the specificity of our antibody in the new Figure S1A.

2. PLA data shown in Figure S1B should be either corroborated by visually counting the centrosomal staining across multiple cells and experimental repeats or removed

R. We agree with the Reviewer. We have now added this important control, counting 46-58 centrosomes in three independent experiments (now reported in Figure S1B).

3. Referencing panels of Fig. S2 in the main text is wrong. Panel C instead of E, E instead of F

R. We thank the Reviewer for pointing out this issue that we amended.

4. The cartoon in Fig. 1D is slightly misleading: it seems myosin VI localizes to the proximal centriole end

R. We agree with the Reviewer and we have now modified the panel accordingly.

5. When referring to MyUb, WWY and RRL: it is not clear where are they positioned within myosin VI. It is also not clear which point mutation affects which domain.

R. We thank the Reviewer for bringing this to our attention. We apologize for not having explained this properly. We have revised the text in the new Figure S2A and its legend.

6. "Interestingly, the SAH domain, while it does not interact with OFD1 per se (Supplementary Figure 2E), appears to be required for the maximum binding (Figure 1E)." Has the contribution of the MIU domain been excluded?

R. We are grateful to the Reviewer for his/her comment. We performed a large number of pulldowns and we did not realize that we had forgotten this point. The missing information is now present in the new panel of Figure 1E. The MIU domain alone is not able to change the binding ability of the minimal construct.

7. Figure 5D: why changing from Cep135 (shown in panels B-C) to Cep164?

R. The answer is purely technical. We decided to combine three antibodies in order to perform two different analyses on the very same samples (Figure 5D and Figure 6B).

Prior studies are referenced appropriately. Text and figures are clear and accurate.

R. We thank the Reviewer for his/her kind words.

(Significance):

In light of the background of this reviewer in centrosome biology, I think that this manuscript will clearly advance the field of cell biology conceptually, deepening in particular the relationship between centrosomes and the actin cytoskeleton, centriolar satellites and ciliogenesis. Better defining the relationship between the diverse phenotypes caused by myosin VI knockdown among each other will undoubtedly boost the overall impact of the manuscript.

R. We thank the Reviewer for his/her kind words and for the experiments suggested that undoubtedly boosted our conclusions.

Reviewer #3 (Evidence, reproducibility and clarity):

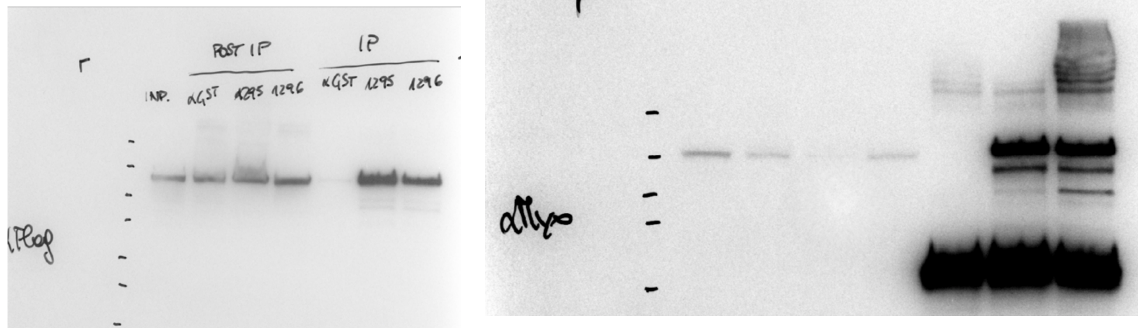
The paper by Magistrati et al. identifies OFD1 as a new interactor of myosin VI. They show that myosin VI regulates the localisation of OFD1 at centrioles. This work links myosin VI to the formation of distal appendages and the primary cilium. They have performed a variety of microscopy techniques, pulldowns and proteomics across multiple cells lines to identify the interaction between myosin VI and OFD1, along with the impact of the interaction.

MAJOR COMMENTS

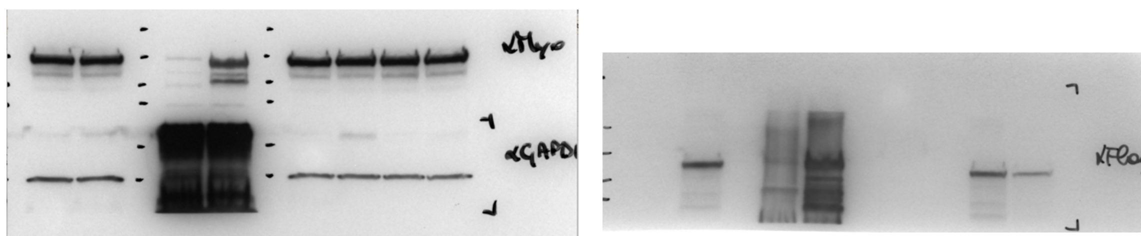
Figure 1: There are concerns with panel B and C which need to be addressed before further processing of this manuscript. The shape/profile of the band staining is essentially identical between myosin VI and Flag-OFD1 in panel B. The same is seen in C, moreover, the second band present in the myosin VI gel is also seen in the Flag-OFD1 lane (just cropped more than myosin VI). As it stands, I believe these are the same samples.

R. An established routine in our lab is to run first the anti-prey and then the anti-bait, in order to avoid a possible cross-reaction during reblotting of the same filter. We are reporting here (as well as in the data source) the uncropped version of the two panels that clearly demonstrated the specificity of our antibodies. FLAG-OFD1 runs faster than endogenous myosin VI.

Panel B, IP anti-myosin VI



Panel C, IP anti-FLag



A range of different cells lines are used throughout the study. It would be useful to show the expression level of myosin VI across these different cells and what isoforms are expressed. This will impact the understanding of results arising from the different experiments/cell lines.

R. True, we did the proteomics approach using different cell lines on purpose, to evaluate common and strong candidate interactors. We have now added the requested experiment and results are reported as a new panel in Figure S1A,B. No major difference was detected regarding the expression level of OFD1, while a clear myosin isoform selective expression is visible among the cell lines. This result further confirmed our data that the interaction is isoform-independent.

It is not clear why proteomics has been performed in MDA-MB-231, HeLa, MCF10a and Caco-2 cells and then pull downs were performed in HEK cells? It would be better/clearer to perform work consistently across different cells lines - especially if attempting to confirm interactions.

R. HEK cells are able to express exogenous constructs at a very high level with a low-cost transfection method. This favors a good and reproducible expression of the different constructs that should be compared.

Point mutations are referred to in Sup Fig 2C/D - I think this is 2D/2E?

R. We thank the Reviewer for pointing out this mistake that we have now edited.

When performing these pulldowns based on transfection of different domains, how successful is the transfection? How variable are the transfections between constructs? Trying to compare pulldown efficiency can be difficult following over-expression of different constructs.

R. We totally agree with these comments and for this reason we used HEK cells. Our conclusions are based on multiple repetitions of the same experiment and we are actually quite satisfied with the comparable level of expression we obtained for the various constructs of myosin VI (Figure S2B-D). More difficult was to express the OFD1 constructs at the same level (Figure 1F), but all of the experiments that we performed invariably confirmed that the interaction is not mediated by the LISH domain or the LIR motif. We are currently running AlphaFold 2 predictions to model the interaction between the two proteins that appears to occur via their coiled-coil regions.

The finding that OFD1 does not interact through the RRL or WWY motifs is interesting for understanding wider interactions within myosin VI interactome. It is stated that the conformation of the tail will likely impact the interaction. It would be useful to investigate the point further to understand the interaction between both proteins.

R. We agree with the Reviewer that mapping the interaction surface between the two proteins will be interesting and for this reason we made a strong effort to produce and crystallize the minimal complex. Unfortunately, OFD1 coiled-coil region resulted to be an insoluble and 'difficult' protein for biochemical studies and this precluded further investigations.

In relation to this point, are you confident that the domains used in this study are correctly folded - therefore able to interact fully with OFD1?

R. Regarding myosin VI, we are totally confident about our domains. We previously published a few papers in collaboration with Kylie Walters, NMR expert (Wollscheid et al. 2016, He et al 2016, Biancospino et al, 2019). The folding of our fragments was studied by NMR and thanks to that we actually discovered that the myosin VI RRL/AAA mutations or even the single arginine mutation (R1117A) were able to abrogate the folding of the entire MyUb domain (He et al, 2016).

There are no images of endogenous myosin VI and OFD1. These should be added to confirm that the interactions are real from a spatial perspective in the cell lines. Overall, it would be useful to show how myosin VI in relation to the structures/images shown in the other figures.
R. We would love to have this possibility, but unfortunately both antibodies are of rabbit origin. Moreover, the anti-myosin VI antibody appears not suitable for super resolution studies as, due to the wide distribution of the protein inside the cell, the signal/noise ratio is too high.

The term "bad behaviour of the shorter construct" needs to be better defined so that the problem is clearly articulated.
R. We apologies for this 'bad' definition. Unfortunately, shorter constructs of the coiled-coils region of OFD1 were barely soluble and this precluded further structural studies. We revised the text to better explain this concept.

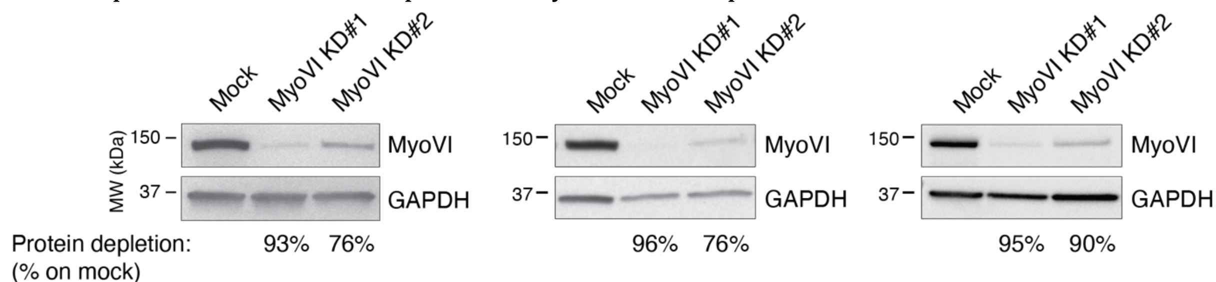
Sup Fig 1A: Myosin VI KD still leads to significant myosin VI staining and Pcnt colocalization - how specific is this signal?
R. Our initial experiment was performed in KD conditions and, as it was mentioned in the legend, myosin depletion was not sufficient to abrogate myosin VI signal at the centriole, thus impeding definitive conclusion on specificity. We have now repeated the experiment in A549 KO cells and optimized our IF protocol, as reported in the new Figure S1A.

When switching cell lines e.g. to hTERT-RPE1, the logic for this is clear but it is important to establish if the interactions see in the other cells lines also occur here.
R. We agree with the Reviewer. We have now performed co-immunoprecipation of endogenous proteins in RPE cells and added this important result in the new Figure 2A.

How does the expression level of both proteins vary?
R. As stated before, we performed the requested experiment and the results are reported in the new Figure S1A.

Sup Fig 3: MyoVI KD2 is very poor according to the western blot yet the impact on centrosome-PM distance is significant. It would be better to show the images of the cells where myosin VI is also stained to clarify if there is heterogeneity within the cell population. As it stands, it appears that a moderate knockdown of Myosin VI causes this impact in centrosome-PM distance? Moreover, N=2 experiments is low for judging significance when the KD is this poor. In relation to the above point, Western blots should be shown for all KDs for the experiments.

R. To address the concern expressed by this Reviewer, we have performed a third experiment and an accurate quantification of the data that are now reported together with the original pictures showing the level of heterogeneity within the cell population. We respectfully disagree with the Referee on considering MyoVI KD2 *poor*, as we measured a depletion between 76 to 90 % in the three experiments. We report here below as well as in the Supplementary Data file the western blots of the three experiments that show reproducibility of our KD experiments.



With respect to the FRAP/Fig4, can you compare the change in mobile fraction in 4C/D to the amount of increased staining of OFD1 upon MyoVI KD in 4A?

R. This is an interesting point, but very difficult to address. Staining of Figure 4A is related to the endogenous protein while the evaluation of the mobile fraction in Figure 4C/D has been performed in GFP-OFD1 overexpressed bulk population, thus with heterogenous levels of expression among the cells. As such, a direct comparison is impossible to perform. Moreover, in this latter context, to avoid additional caveats, we considered single cells, in which the initial expression of OFD1 was comparable as reported here on the right.

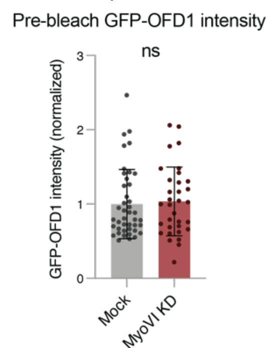


Fig 5: Panel D shows a highly pixelated image - is the same bar correct and equivalent to panels B and C?

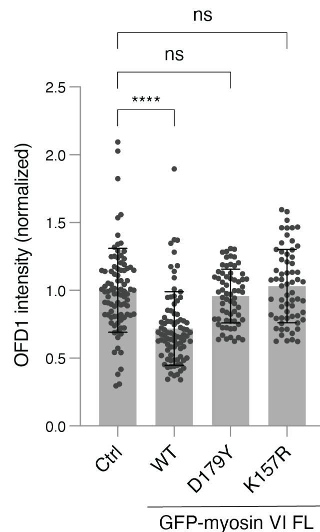
R. We apologize for the mistake. We erroneously used a low-resolution picture when we mounted the panels. This has now been corrected.

Discussion states that cell cycle arrest was not seen in cancer cell lines - where is this data in the manuscript?

R. We apologize for not having reported these data and explained this important point in the original version of the manuscript. We have now added proliferation experiments performed on HeLa and Caco-2 cancer cells depleted of myosin VI. See also our answer to point 3 of Reviewer #1.

Overall, can you state how myosin VI is involved? How it is working? Is it transporting OFD1 or stabilising its interaction/crosslinking it to centrioles? Does myosin VI ATPase, actin binding mutants cause similar impact on OFD1? The work here shows that there is an interaction and KD causes an impact but it would be interesting to see a direct functional response/ or at least strong hints towards a functional role.

R. We agree with the Reviewer that all these questions are extremely interesting. To gain additional insights, we performed an overexpression experiment in RPE cells using GFP-tagged constructs, the results are reported here below.



Overexpression of wild-type myosin VI construct appears to have the opposite effect on OFD1 level while ATPase and actin binding mutants show no impact. Thus, it seems that mutants do not act as dominant negative in this context. The degree of difference is, however, limited (average value WT= 0,72; D179Y=0.96; K157R=1.03); as the efficiency of the transfection in this cell line is limited. Solidifying this data is therefore quite demanding.

While we are actively working on furthering our understanding of the mechanistic role exerted by myosin VI on OFD1, we believe that dissecting this function in greater detail will require a large amount of effort that is beyond the scope of the current manuscript, which is first to identify the function of myosin VI at centrioles and in cilium formation.

MINOR COMMENTS

The introduction could benefit from more detail on myosin VI - such as types of isoforms, how myosin VI is regulated. This will provide the reader with more context in relation to the experiments performed and results.

R. We respectfully disagree with the Referee on this point. In the introduction we focus our attention on the OFD1, centriole biology and primary cilia as the main phenotypes that we analyze in the paper are related to these. As also pointed out by this Reviewer, our breakthrough is related to the functional impact of myosin VI, while the mechanism of regulation remains unclear. Thus, we found it quite difficult to identify the relevant features to cite in the introduction among the vast array of published literature on myosin VI.

In the first section of the results: Short and long myosin VI isoforms are mentioned but not defined - please clarify.

R. We apologize for the inaccurate description of the isoforms that we actually fully characterized in our previous publications (Wollscheid et al 2016; He et al 2016). We modified the results section and we edited the legend of Figure S1, adding additional data on myosin VI isoforms.

Full uncropped western blots should be shown as a supplementary figure.

R. We prepared an uncropped version of all the blots that now are added as Supplementary Data file.

(Significance):

This paper presents and interesting finding regarding a link between myosin VI and OFD1. The authors tie this observation to the formation of distal appendages and the primary cilium. As it stands, this work provides evidence for the interaction and an impact of myosin VI KD upon OFD1 localisation. But, very

little information is given on the functional role of myosin VI in this process. Therefore, this work will provide a foundation for further studies to define the role of myosin VI in this process.
My field of expertise: Myosin VI regulation and interactions, biochemistry, biophysics and imaging.
R. We thank this Reviewer for his/her numerous useful suggestions that we have now implemented to improve our study.

1st Editorial Decision 19th Nov 2021

Dear Simona,

Thank you for submitting your revised manuscript, which was previously reviewed at Review Commons. I have now heard back from one of the original referees. The referee finds that the manuscript was significantly improved after revision and recommends publication. Before I can accept the manuscript, I need you to address some editorial points below:

• Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide)
• Main figures should be uploaded as individual high-resolution figure files. Supplementary figures can be renamed as EV Figures, in which case they would need to be uploaded as individual files. Please note that for technical reasons we can accommodate maximum 5 EV Figures. Currently, there are 6 supplementary figures. You can either combine two of the figures to reduce the figure number to 5, or you can compile all in an Appendix, with their legends and a table of contents. Please remember to update figure callouts accordingly.
• Please upload the manuscript text in word format.
• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• Please make sure that the dataset PXD026697 is publicly accessible.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• During our routine figure checks we notice that mock panel of Figure 3G is re-used in Figure S5B. This is only allowed if all panels are derived from the same experiment, in which case it needs to be spelled out in the figure legends.
• Supplementary Table should be renamed as "Dataset EV" and needs a legend.
• Please upload the source data as one file per figure.
• The antibodies table in the Materials&Methods section needs a title and a description.
• Materials&Methods section needs to be moved after Discussion.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper (max 35 words) and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels. For example, you can adapt Figure 10 for this.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have taken into serious consideration and adequately addressed all the points that I raised. I think that the manuscript can be published in EMBO Rep without any further delay.

Referee #2:

The authors have taken significant steps to address the points raised during the initial review by Review Commons. I am satisfied with the changes and additional data which has been presented and/or the justifications given in response.

This is a significant body of work which covers multiple techniques which I hope will be published promptly.

The authors have addressed all minor editorial requests.

1st Revision - Editorial Decision 8th Dec 2021

Dear Simona,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2021-54160V2 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ➔

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:Elisa Magistrati, Simona Polo

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2021-54160

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

B- Statistics and general methods

	Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to determine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data points were excluded in this work. All data points were represented in the figures and used in statistical analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	not applicable
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Researchers were blinded to group allocation during immunofluorescence experiments. For other experiments, we relied upon replication and confirmations of results by at least 2 independent techniques or investigators when possible.
4.b. For animal studies, include a statement about blinding even if no blinding was done	not applicable
5. For every figure, are statistical tests justified as appropriate?	When needed, a full description of the statistical parameters including central tendency (e.g. means) or other basic variation (e.g. standard deviation) is reported together with the exact sample size (n) for each experimental group/condition and the statistical test used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	not applicable
Is there an estimate of variation within each group of data?	Yes

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Done
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Done

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	not applicable