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Cep78 controls centrosome homeostasis by inhibiting EDD-DYRK2-DDB1^{VprBP}

Delowar Hossain, Yalda Javadi Esfehani, Arindam Das, and William Tsang

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Transaction Report:

1st Editorial Decision 07 April 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, two referees acknowledge the interest of the findings and support the publication of your study. However, referee 2# is more critical and raises several critical points and has also technical or experimental concerns. Also referee #3 has raised a number of points. Thus, two referees point out major concerns and have a number of suggestions for how the study should be strengthened, and I think that all of this should be addressed. In particular, the comparison with other E3 ligases (first point of ref #2), the validation that CP110 is a substrate of EDD-DBB1-VprBP (second point of ref #2), the concerns regarding the siRNA experiments (fifth and sixth point of ref #2) and point 1 of referee #3 are of particular importance.

Given the positive assessment of two referees and the constructive comments by referee #2 and #3, we would like to invite the revision of your manuscript with the understanding that all referee concerns (as detailed in their reports) must be addressed in a point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

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Referee #1:

In recent years, there has been accumulating data in support of a major role for the E3 ligase EDD-DYRK2-DDB1VprBP in cell cycle control, specifically in mitotic progression. In contrast, there is an incomplete understanding of how or if EDD-DYRK2-DDB1VprBP activity is regulated through G1and S phases of the cell cycle. In this report, Hossain et al. now provide multiple lines of evidence in support of a novel mechanism through which a centriolar pool of EDD-DYRK2-DDB1VprBP activity is silenced during the G1/S transition by recruitment to the distal ends of both centrioles of the centrosome by Cep78, a novel Cep76 interacting protein. Interestingly, silencing of Cep78 inappropriately induced initiation of the ciliogenic program by inducing the degradation of CP110 by EDD-DYRK2-DDB1VprBP ligase. One very exciting aspect of this report is that it has uncovered a novel layer of regulation of CP110 ubiquitination in cycling cells.

This report is quite thorough and complete, and the quality of data is uniformly high. It is well controlled and all conclusions are supported by the data. It will be of great interest to the general readership of EMBO Reports, and I enthusiastically recommend publication with no further revisions.

Referee #2:

The manuscript by Tsang et al suggests that the uncharacterized centrosomal protein Cep78 inhibits the degradation of CP110 by the EDD-DDB1-VPRBP ubiquitin ligase. There are some novel and interesting findings within this manuscript, however some important controls are missing, making it difficult to interpret the data. Thus, at this stage, I cannot recommend publication in Embo Reports. Some suggestions for improvement of the manuscript are listed below.

<u>Major concerns:</u>

- CP110 ubiquitination is known to be controlled by other ubiquitin ligases (Cyclin F and Neurl4) and reversed by the deubiquitinase (USP33). The authors should address whether Cep78 can inhibit the ubiquitination of CP110 by Cyclin F or Neurl4 and compare the contributions of these two ligases (Cyclin F and EDD-DDB1-VPRBP) to the turnover of CP110. Do the different ubiquitin ligases operate at different cell cycle stages?

- Further validation is needed to prove that CP110 is a substrate of EDD-DDB1-VprBP. This includes demonstrating:

- endogenous VprBP can interact with endogenous CP110,
- in vitro ubiquitination of CP110 using purified components,
- that the half life or steady state levels of CP110 are increased when VprBP is silenced by siRNA

- The authors show that Cep78 interacts with the EDD-DDB1-VprBP E3 ubiquitin ligase and conclude that Cep78 is not a substrate of VprBP. Further experiments should be completed to conclude that Cep78 is not a substrate of VprBP. For example, the authors should test whether Cep78 is a substrate of EDD-DDB1 in mitosis (when Cep78 levels are downregulated). The authors should test whether the Cep78 D262A mutant is stable in mitosis and whether Cep78 is stable in mitosis when VprBP is depleted by siRNA.

- The authors do not explain how Cep78 might inhibit the ubiquitination of non-centrosomal substrates (e.g. TERT). Is there a pool of Cep78 at telomeres? Is EDD-DDB1-VPRBP sequestered at the centrosome by Cep78 and blocked from interacting with TERT?

- A general concern is that the siRNA experiments in the manuscript only show one siRNA condition. It is standard practice that multiple siRNAs should be tested, and a rescue/complementation experiment performed for the key figures (fig 4d, 5g,6a).

- The effect of Cep78 siRNA on CP110 levels may be indirect, given that the levels of many

centrosomal proteins are dependent on the levels of other centrosomal proteins (i.e. siRNA for one centrosomal protein results in down-regulation of other centrosomal proteins), Thus, the Cep78 D262A mutant (that does not bind VprBP) is an important control that would allow the authors to confirm that Cep78 functions through the VprBP pathway. This control should be used in figure 5E (ubiquitination of CP110), and 6C,D or 6G,H (centriole elongation phenotype).

There is no experiment proving that Dyrk2 is the pertinent kinase (e.g. DYRK2 siRNA or DYRK2 inhibitor).

<u>Minor points:</u>

- The authors say that EDD, DYRK2 and VprBP localize the centrosome in ~ 30% of cells. The authors should establish if this occurs in a cell cycle specific manner.

- White boxes surrounding the region of interest should be used to mark the magnified area in figures.

- The VprBP is defined as "Viral Protein R" in the text, but should be defined as Viral Protein R Binding Protein. The alternative name DCAF1 should be mentioned in the introduction.

- The title is very non-specific in its current form. A more detailed title could be, for example, Cep78 maintains centriole length by counteracting EDD-DYRK2-DDB1 VprBP-mediated ubiquitination and degradation of CP110.

- Mass Spec results (Figure 2A) should include more details, e.g. # of spectra, # unique peptides, relative size of protein.

- Figure S2B. It is very difficult to see the interaction between Cep78 and DDB1 in the chosen exposure.

Referee #3:

<u>Comments to authors:</u>

In this manuscript, Hossain et al. demonstrate a novel mechanism involving Cep78, CP110 and EDD-DYRK2-DDB1VprBP E3 ligase complex that regulates centriole length and cilia assembly. They showed that all these components are centrosomal proteins and that Cep78 interacts with the E3 complex via VprBP receptor subunit and specifically prevents ubiquitin transfer onto CP110 without changing the subunit composition of the complex. Down regulation of Cep78 thereby destabilizes CP110, resulting in centriole elongation and cilia assembly. The ubiquitination of CP110 requires its phosphorylation by DYRK2 and binding to VprBP.

Overall, the data supporting these findings are mostly clean and compelling. I enjoyed reading this paper and the mechanism proposed is interesting, novel and important in the basic and clinical field. This study would be appropriate for the journal, provided that the authors can address the issues outlined below.

<u>Major Criticisms:</u>

1) The data supporting VprBP-mediated interaction between Cep78 and EDD-DYRK2-DDB1VprBP is not sufficient. It is only shown in Figure S2D with DDB1 immunoblotting, which is not vey convincing. Although authors demonstrated direct binding of Cep78 with VprBP, but not others in Figure 2E, it is very possible that the single subunit of the complex may not work in vitro. Because this is important point, inhibition of co-precipitation of EDD, DYRK2 and DDB1 in Cep78-immunoprecipitation by VprBP knockdown should be addressed.

2) Evidence for the phosphorylation of CP110 by DYRK2 is not shown. It is only suspected by

mutational analyses. In vitro phosphorylation assay is recommended to address this point. Alternatively, inhibition of the in vivo phosphorylation by DYRK2 knockdown should at least be shown.

Minor criticisms:

1) The interaction between endogenous Cep78 and CP110 is not shown. Is this interaction weak or transient in physiological condition?

2) Is Cullin4 indicated in Figures 2 and 5 Cullin4A, 4B or both?

3) Positive control for the fractions other than Fraction-5 is better to be included in Figure 3A.

4) It is not clear how the overexpression of only one subunit of the E3 complex (such as EDD and DDB1) is able to upregulate the activity of entire complex in Figure 5F. Explanation may need for the findings.

5) CP110 phosphorylation in Figure 7E is not clear.

1st Revision - authors' response 30 June 2016

We thank the Reviewers for the positive assessment of our manuscript and the constructive comments. Their rigorous review has substantially improved the quality and presentation of our manuscript.

Reviewer #1:

In recent years, there has been accumulating data in support of a major role for the E3 ligase EDD-DYRK2-DDB1VprBP in cell cycle control, specifically in mitotic progression. In contrast, there is an incomplete understanding of how or if EDD-DYRK2-DDB1VprBP activity is regulated through G1and S phases of the cell cycle. In this report, Hossain et al. now provide multiple lines of evidence in support of a novel mechanism through which a centriolar pool of EDD-DYRK2-DDB1VprBP activity is silenced during the G1/S transition by recruitment to the distal ends of both centrioles of the centrosome by Cep78, a novel Cep76 interacting protein. Interestingly, silencing of Cep78 inappropriately induced initiation of the ciliogenic program by inducing the degradation of CP110 by EDD-DYRK2-DDB1VprBP ligase. One very exciting aspect of this report is that it has uncovered a novel layer of regulation of CP110 ubiquitination in cycling cells. This report is quite thorough and complete, and the quality of data is uniformly high. It is well controlled and all conclusions are supported by the data. It will be of great interest to the general readership of EMBO Reports, and I enthusiastically recommend publication with no further revisions.

We thank Reviewer #1 for pointing out that our manuscript "is quite thorough and complete" and "will be of great interest to the general readership of EMBO Reports."

Reviewer #2:

The manuscript by Tsang et al suggests that the uncharacterized centrosomal protein Cep78 inhibits the degradation of CP110 by the EDD-DDB1-VPRBP ubiquitin ligase. There are some novel and interesting findings within this manuscript, however some important controls are missing, making it difficult to interpret the data. Thus, at this stage, I cannot recommend publication in Embo Reports. Some suggestions for improvement of the manuscript are listed below.

Major concerns:

1) CP110 ubiquitination is known to be controlled by other ubiquitin ligases (Cyclin F and Neurl4) and reversed by the deubiquitinase (USP33). The authors should address whether Cep78 can inhibit

the ubiquitination of CP110 by Cyclin F or Neurl4 and compare the contributions of these two ligases (Cyclin F and EDD-DDB1-VPRBP) to the turnover of CP110. Do the different ubiquitin ligases operate at different cell cycle stages?

This Reviewer had raised few important points. To address whether Cep78 can inhibit the ubiquitination of CP110 by Cyclin F or Neurl4, we first demonstrated that CP110 ubiquitination can be independently triggered by VprBP, Cyclin F or Neurl4 (Appendix Figure S5A). Next, we showed that while co-expression of Cep78 with VprBP suppresses CP110 ubiquitination, co-expression of Cep78 with Cyclin F or Neurl4 does not (Appendix Figure S5A), suggesting that Cep78 can only suppress EDD-DYRK2-DDB1VprBP. We also compared the contributions of these E3 ligases to the turnover of CP110. We found that while CP110 protein levels are decreased by expression of VprBP, they can be further reduced by co-expression with Cyclin F or Neuralized4 (Appendix Figure S5B). Together, our data suggest that these three E3 ligases likely function in a mechanistically distinct manner to regulate CP110 ubiquitination.

Cyclin F is known to destabilize CP110 in G2 phase. Neurl4, on the other hand, may act on CP110 throughout the cell cycle. In our work, we found that the ubiquitination activity of EDD-DYRK2-DDB1VprBP at the centrosome is controlled by Cep78. We believe that EDD-DYRK2-DDB1VprBP functions in mitosis when Cep78 levels are low. Consistent with this notion, this E3 ligase has also been suggested to function in mitosis (Nishi and Lin, Dev Biol (2005); Munoz et al., Cell Cycle (2007); Maddika and Chen, Nat Cell Biol (2009); Jung et al, JBC (2013)). Taken together, these three E3 ligases likely operate at different cell cycle stages. We have now included a sentence in the Discussion postulating that EDD-DYRK2-DDB1VprBP likely operates in mitosis.

2) Further validation is needed to prove that CP110 is a substrate of EDD-DDB1-VprBP. This includes demonstrating:

- endogenous VprBP can interact with endogenous CP110
- in vitro ubiquitination of CP110 using purified components
- that the half life or steady state levels of CP110 are increased when VprBP is silenced by siRNA

The endogenous interaction between VprBP and CP110 has been confirmed by co-immunoprecipitation experiments in both directions. This data is presented in new Figure 5A. Old Figure 5A becomes new Appendix Figure S3A, and old Figures S8A-B become new Appendix Figures S4A-B.

We have performed in vitro ubiquitination experiments using purified ubiquitin, E1, E2, EDD, DYRK2, DDB1, VprBP and CP110 in an ubiquitination reaction buffer. Indeed, CP110 was robustly ubiquitinated when all components were present in the reaction mixture (new Figure 5F). In contrast, CP110 was not ubiquitinated when one component was missing (new Figure 5F) or when purified Cep78 was also added to the reaction (new Figure 5F). Old Figure 5F becomes new Appendix Figure S3B. As suggested, we also depleted VprBP by siRNA in HeLa cells and showed that the protein levels of CP110 increase. This data is shown in new Figure 5I.

3) The authors show that Cep78 interacts with the EDD-DDB1-VprBP E3 ubiquitin ligase and conclude that Cep78 is not a substrate of VprBP. Further experiments should be completed to conclude that Cep78 is not a substrate of VprBP. For example, the authors should test whether Cep78 is a substrate of EDD-DDB1 in mitosis (when Cep78 levels are downregulated). The authors should test whether the Cep78 D262A mutant is stable in mitosis and whether Cep78 is stable in mitosis when VprBP is depleted by siRNA.

We have tested whether Cep78 is substrate of EDD-DYRK2-DDB1VprBP in mitosis by monitoring ubiquitination of Cep78 in synchronized mitotic cells (obtained by treating cells with 40 ng/ml nocodazole for 24 hours) that express either myc or myc-VprBP. The levels of Cep78 ubiquitination were found to be similar between the two conditions, suggesting that VprBP does not trigger Cep78 ubiquitination in mitosis. This data is presented in new Figure EV5C. Old Figures S5C-D become new Figures EV5D-E.
To address whether the VprBP-binding mutant of Cep78 is stable in mitosis, we have attempted to generate stable HEK293, RPE and HeLa cell lines expressing Flag (control), Flag-Cep78 wild type or Flag-Cep78D290A and examined the levels of recombinant protein across the cell cycle. We

chose to use the D290A mutant instead of D262A because we have previously performed additional studies with this mutant (old Figure S6C/new Appendix Figure S1C). Unfortunately, we were not able to obtain sustainable expression of Flag-Cep78 wild type or mutant after 2-3 weeks of selection, thus precluding further experimentation. Instead, we have examined and compared the levels of ubiquitination of Cep78 wild type and mutant proteins in mitosis. We found that Cep78 wild type and the D290A mutant exhibit comparable levels of ubiquitination in mitosis (Figure EV5G), suggesting that the stability is very similar for wild type and mutant proteins and that both proteins are likely to be down-regulated in mitosis.

We have previously found that depletion of VprBP by siRNA does not affect the steady-state levels of Cep78 in asynchronous cells (Figure 4B). This finding has now been extended to mitotic cells (new Figure EV5F). All together, we strongly believe that these results further strengthen the notion that Cep78 is not a substrate of VprBP.

4) The authors do not explain how Cep78 might inhibit the ubiquitination of non-centrosomal substrates (e.g. TERT). Is there a pool of Cep78 at telomeres? Is EDD-DDB1-VPRBP sequestered at the centrosome by Cep78 and blocked from interacting with TERT?

We have only been able to detect Cep78 at the centrosome but not at telomeres. On the other hand, components of the EDD-DYRK2-DDB1VprBP are localized to the centrosome and telomeres, and the three EDD-DYRK2-DDB1VprBP substrates identified so far are either nuclear (TERT) or centrosomal (katanin p60 and CP110) proteins. These observations suggest that there are probably two pools of EDD-DYRK2-DDB1VprBP in cells and only the centrosomal pool is regulated by Cep78. Although it is possible that Cep78 sequesters VprBP at the centrosome and blocks it from interacting with TERT, we find this scenario highly unlikely since neither depletion nor overexpression of Cep78 affects the protein levels or localization of VprBP (old Figure S5A/new Figure EV5A; old Figures S6A-B/new Appendix Figures S1A-B). Rather, the inhibition of TERT ubiquitination by Cep78 (old Figures S7B and S7D/new Appendix Figures S2B and S2D) might be attributed to the use of cellular extracts in our in vivo ubiquitination assays where cells were broken open and cellular contents were mixed. Nevertheless, our findings that Cep78 can inhibit ubiquitination of TERT, but not a CRL4VprBP substrate MCM10 (which is also a nuclear protein; old Figures S7E-F/new Appendix Figures S2E-F) is intriguing because it offers further biochemical proof that Cep78 is a specific inhibitor of EDD-DYRK2-DDB1VprBP. We have now revised the following sentence in page 11: "To unambiguously and biochemically proof that Cep78 regulates EDD-DYRK2-DDB1VprBP but not CRL4VprBP, we found that depletion of Cep78 leads to enhanced ubiquitination of katanin p60 and TERT...."

5) A general concern is that the siRNA experiments in the manuscript only show one siRNA condition. It is standard practice that multiple siRNAs should be tested, and a rescue /complementation experiment performed for the key figures (fig 4d, 5g,6a).

The experiment in Figure 4D was performed with Cep78 siRNA oligo 1. Identical results were obtained with two additional Cep78 siRNA oligos (oligos 2 and 6) and these results are presented in new Appendix Figure S1D.
The experiments in Figure 5G were conducted with Cep78 siRNA oligo 1. We have since repeated and reproduced the data with two additional Cep78 siRNA oligos (oligos 2 and 6). In addition, we have shown that decreased CP110 protein levels due to depletion of Cep78 with a siRNA oligo that targets the 3iUTR of Cep78 mRNA (oligo 7) can be rescued by expression of Flag-Cep78. These data are presented in new Appendix Figures S3C-D.
The experiments in Figures 6A-B were also performed with Cep78 siRNA oligo 1. We have now confirmed these results with two additional Cep78 oligos (oligo2 and 6). In addition, we have shown that the centriole elongation phenotype due to depletion of Cep78 with oligo 7 can be rescued by expression of exogenous Cep78. These new data are added to Appendix Figures S6A-D.

6) The effect of Cep78 siRNA on CP110 levels may be indirect, given that the levels of many centrosomal proteins are dependent on the levels of other centrosomal proteins (i.e. siRNA for one centrosomal protein results in down-regulation of other centrosomal proteins), Thus, the Cep78 D262A mutant (that does not bind VprBP) is an important control that would allow the authors to

confirm that Cep78 functions through the VprBP pathway. This control should be used in figure 5E (ubiquitination of CP110), and 6C,D or 6G,H (centriole elongation phenotype).

We have now shown that expression of the VprBP-binding mutant of Cep78 (D290A), unlike wild type Cep78, does not suppress CP110 ubiquitination. This data is added to Figure 5E.
We also showed that the centriole elongation phenotype induced by Cep97 loss cannot be effectively suppressed by D290A. This data is added to Figures 6C-D.
Furthermore, Cep78D290A was less efficient in suppressing cilia formation than Cep78 wild type in quiescent RPE-1 cells, as shown in Figures 6G-H.

7) There is no experiment proving that Dyrk2 is the pertinent kinase (e.g. DYRK2 siRNA or DYRK2 inhibitor).

We have performed in vitro kinase assays using purified proteins and demonstrated that CP110 can be phosphorylated by DYRK2 alone or EDD-DYRK2-DDB1VprBP in vitro. This data is presented in Figure 7B. Old Figures 7B-G becomes new Figures 7C-H.

Minor points:

1) The authors say that EDD, DYRK2 and VprBP localize the centrosome in ~ 30% of cells. The authors should establish if this occurs in a cell cycle specific manner.

We have examined the localization of EDD, DYRK2 and VprBP in G1, S, G2 and M phases and found that these proteins localize to the centrosome in about 5-65% of cells during the cell cycle. This new data is shown in Figure 3C. Old Figures 3C-E become new Figures 3D-F.

2) White boxes surrounding the region of interest should be used to mark the magnified area in figures.

We have now added white boxes to the figures.

3) The VprBP is defined as "Viral Protein R" in the text, but should be defined as Viral Protein R Binding Protein. The alternative name DCAF1 should be mentioned in the introduction.

Thank you for pointing out the mistake. We have made the correction and mentioned DCAF1 in the introduction.

4) The title is very non-specific in its current form. A more detailed title could be, for example, Cep78 maintains centriole length by counteracting EDD-DYRK2-DDB1 VprBP-mediated ubiquitination and degradation of CP110.

We believe that the title in its current form best reflects the biological function of Cep78 at the centrosome, which is to regulate ubiquitination of EDD-DYRK2-DDB1VprBP substrates and centrosome homeostasis. Although we have identified CP110 as a new substrate of EDD-DYRK2-DDB1VprBP, it is clear that this enzyme can ubiquitinate at least one other centrosomal substrate katanin p60. Given that there is more than one centrosomal substrate and that CP110 is a multi-functional protein which controls more than just centriole length, a more general title is appropriate. Nevertheless, we would be happy to modify the title at the discretion of the Editor.

5) Mass Spec results (Figure 2A) should include more details, e.g. # of spectra, # unique peptides, relative size of protein.

This information is now provided in Figure 2A.

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6) Figure S2B. It is very difficult to see the interaction between Cep78 and DDB1 in the chosen exposure.

This blot has now been replaced with a better one. We thank this Reviewer for a constructive critique, which has led to a substantially improved manuscript.

Reviewer #3:

In this manuscript, Hossain et al. demonstrate a novel mechanism involving Cep78, CP110 and EDD-DYRK2-DDB1VprBP E3 ligase complex that regulates centriole length and cilia assembly. They showed that all these components are centrosomal proteins and that Cep78 interacts with the E3 complex via VprBP receptor subunit and specifically prevents ubiquitin transfer onto CP110 without changing the subunit composition of the complex. Down regulation of Cep78 thereby destabilizes CP110, resulting in centriole elongation and cilia assembly. The ubiquitination of CP110 requires its phosphorylation by DYRK2 and binding to VprBP. Overall, the data supporting these findings are mostly clean and compelling. I enjoyed reading this paper and the mechanism proposed is interesting, novel and important in the basic and clinical field. This study would be appropriate for the journal, provided that the authors can address the issues outlined below.

We thank the Reviewer for his/her positive assessment of our manuscript.

Major Criticisms:

1) The data supporting VprBP-mediated interaction between Cep78 and EDD-DYRK2-DDB1VprBP is not sufficient. It is only shown in Figure S2D with DDB1 immunoblotting, which is not vey convincing. Although authors demonstrated direct binding of Cep78 with VprBP, but not others in Figure 2E, it is very possible that the single subunit of the complex may not work in vitro. Because this is important point, inhibition of co-precipitation of EDD, DYRK2 and DDB1 in Cep78-immunoprecipitation by VprBP knockdown should be addressed.

We agree with the Reviewer that this is a very important point. We transfected HEK293 cells with non-specific or VprBP siRNA and performed immunoprecipitation experiments with an anti-Cep78 antibody. Indeed, we showed that EDD, DYRK2 and DDB1 do not efficiently co-immunoprecipitate with Cep78 when VprBP is depleted (new Figure EV2D), thereby confirming our in vitro results (Figure 2E). Old Figure S2D was removed.

2) Evidence for the phosphorylation of CP110 by DYRK2 is not shown. It is only suspected by mutational analyses. In vitro phosphorylation assay is recommended to address this point. Alternatively, inhibition of the in vivo phosphorylation by DYRK2 knockdown should at least be shown.

We have performed in vitro kinase assays using purified proteins and demonstrated that CP110 can be phosphorylated by DYRK2 alone or EDD-DYRK2-DDB1VprBP in vitro. This data is added to Figure 7B. Old Figures 7B-G becomes new Figures 7C-H.

Minor criticisms:

1) The interaction between endogenous Cep78 and CP110 is not shown. Is this interaction weak or transient in physiological condition?

We have now presented the interaction between endogenous Cep78 and CP110 in Figure 5A. This interaction seems to be much weaker than the endogenous interaction between Cep78 and VprBP.

Because Cep78 directly binds to VprBP which in turn interacts with its substrate CP110, we speculate that the interaction between Cep78 and CP110 is probably indirect and therefore weak.

2) Is Cullin4 indicated in Figures 2 and 5 Cullin4A, 4B or both?

It is Cullin4A since the antibody was raised against the C-terminal part human Cullin4A. Thank you for pointing this out and we have made all necessary changes in our manuscript and Figures.

3) Positive control for the fractions other than Fraction-5 is better to be included in Figure 3A.

We have already included two centrosomal markers, centrin and -tubulin, as positive controls to show that centrosomes are sedimented into the sucrose gradient and enriched in Fraction 5. Perhaps this Reviewer was referring to a negative control, which we have now included in Figure 3A.

4) It is not clear how the overexpression of only one subunit of the E3 complex (such as EDD and DDB1) is able to upregulate the activity of entire complex in Figure 5F. Explanation may need for the findings.

This is a very interesting question since the phenomenon that overexpression of only one subunit increases the activity of EDD-DYRK2-DDB1VprBP has also been reported previously (Maddika and Chen, Nat Cell Biol (2009) and Jung et al, JBC (2013)). Our interpretation is as follows: EDD, DYRK2, DDB1 and VprBP proteins can exist as individual subunits or assemble into a complex (EDD-DYRK2-DDB1VprBP). Under unperturbed conditions, a dynamic equilibrium is reached between subunit assembly and disassembly. Upon overexpression of one subunit, the equilibrium between subunit assembly and disassembly is disrupted and the reaction shifts toward complex formation, resulting in an increased enzyme activity. Further biochemical and biophysical studies would be needed to test this idea.

5) CP110 phosphorylation in Figure 7E is not clear.

We have replaced the old blot with a new one to better illustrate the point that CP110 phosphorylation is not affected by Cep78. We thank the Reviewer for his/her comments, which have genuinely improved our manuscript.

2nd Editorial Decision 02 August 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have received the enclosed reports on it. As you will see, both referees now in principle support publication of the manuscript. Despite this assessment, the following issues unfortunately preclude publication of this dataset.

We undertake systematic screens for image aberration and text duplication. This screen uncovered a number of digital aberrations. In accordance with our journal guidelines, we requested source data for all figures from you on 14.07.2016.

Thank you for providing digital versions of the source data for the majority of the Western blot panels of your revised manuscript alongside your detailed analysis. You provided source data for 239 panels (Figures 1-7 and Expanded View Figures 1-5) and noted in your response that 41 of these panels had been "incorrectly processed" and that for 48 panels the original blots were not available, precluding further examination. Later, you also provided the source data for the Appendix figures.

We have now analyzed the blots that you sent and, where available, compared them with the data presented in the manuscript. We are sorry to say that the examination did not resolve the

inconsistencies in the datasets that our and your own assessment uncovered.

In our view, these concerns and ambiguities cast doubt on the reliability of the data in this manuscript and consequently we cannot offer to publish your manuscript.

We therefore at this stage formally decline publication of the current dataset.

Yours sincerely,

Achim Breiling and Bernd Pulverer

Referee #2:

I have examined the revised manuscript. I think that the authors have correctly addressed my comments and changed the manuscript accordingly. The authors have performed many follow-up experiments to comprehensively address the concerns raised in the first revision. They provide data demonstrating that Cep78 specifically inhibits VprBP but not other CP110-directed ubiquitin ligases, to validate the interaction between VprBP and CP110, and to demonstrate that Cep78 is not a substrate of VprBP, and to show that DYRK2 is able to phosphorylate CP110.

Referee #3:

Comments to authors: Most of the concerns I pointed out have been cleared and the manuscript has been essentially improved. Minor criticism 3) remains unsolved. Figure 3A lacks control for fractionation to show that the other fractions contain some amounts of proteins.

Re-submission - authors' cover letter 04 November 2016

Thank you for the opportunity to re-submit our revised manuscript. We have now provided source data for all panels. We have also documented all the changes made to the previous version of the manuscript.

For misplaced blots, we have repeated the experiments to verify the accuracy of our previous findings. In some cases, part of the experiment was repeated. In other cases, the entire experiment was repeated. New blots were presented in the current version.

For blots that were aberrantly manipulated, they were done for the purpose of data beautification wherein a certain region of the blot was processed. In order to prevent this from happening in the current version, most blots were now minimally processed. If they were processed, processing was applied to the entire blot by adjusting brightness and contrast only. In some cases, the same blots with minimal/appropriate processing were presented. In other cases, experiments were repeated and new blots were presented.

A number of blots that were neither misplaced nor misprocessed in the previous version have also been replaced with new ones. This is because many blots have been rescanned and we had multiple blots for the same experiment.

For immunofluorsecent images, we were unable to locate the original uncropped images. Thus, all images have been retaken and new images were presented.

In the end, I am happy to inform that all our previous experimental findings have been validated and hence our conclusions remained unchanged. We thank you for your consideration and look forward

to receiving your response.

Letter to the Institution 30 November 2016

Letter to research institution: MONTREAL CLINICAL RESEARCH INSTITUTE (IRCM)

Dr. Tsang has now resubmitted a manuscript in which some of the original source data are included to replace the manipulated images. We have checked these and as far as we can tell, the current data looks unmanipulated, and the information that was removed by the previous image editing would not argue against the validity of the data or the conclusions based on this data. For the other experiments, the data was apparently lost. Instead, Dr. Tsang repeated the experiments (I am unclear if he re-ran the same samples or repeated the whole experiment). These data again at face value look unmanipulated and in principle allow for the same conclusions as those reached previously.

We remain entirely unclear as to the motivations for these manipulations.

We now have to make a binary decision whether to allow publication of the revised dataset or not. The difficulty in making an informed decision is that we have not interviewed Dr. Tsang or the other co-authors, nor have we been able to analyze the labbooks. Without this information, which would be obtained as part of a formal institutional investigation, we cannot be sure why the original data were manipulated nor be formally convinced that the new data are completely trustworthy. Without a formal independent confirmation of the quality of the data, we are not confident to proceed with publication, as this is based entirely on trust that the data are reliable.

We will put this manuscript on hold until we can secure and update from you or someone formally designated by you to investigate on this case.

Yours sincerely,

Bernd Pulverer

Investigation report by the MONTREAL CLINICAL RESEARCH INSTITUTE (IRCM) [excerpt] 23 December 2016

Investigation of the data included in a manuscript submitted to EMBO Reports

Following the email from the EMBO Journal chief editor, Dr. Pulverer, an IRCM internal review committee composed of two recognized scientist was formed to investigate the quality of the data included in a manuscript of Dr. Tsang. This work was recently re-submitted to EMBO Reports, in the form of an extensively revised manuscript, following the detection of image alterations in the original submission.

Results of the investigation:

Note: Dr. Tsang was highly cooperative and ensured that we had unrestricted access to all of the original biochemical data included in the manuscript in the format of scanned full blots (assembled into an extensive 72 pages PDF document containing 4.4Mb of data) throughout the investigation. We also obtained access to a secure drive where the entire raw fluorescence images were deposited and allowed their viewing.

The committee went systematically through the entire individual blots and assessed how the final figures were assembled. We did a similar careful analysis of all fluorescence images by comparing them to the final figures. We intentionally took an approach of being over critical and this led us to raise a number of points for discussion with Dr. Tsang, including requesting actual original films in addition to the PDF documents. Globally, we found that all the data used to generate the figures included in the revised manuscript are legitimate: the data has not been manipulated or embellished as far as we could see. We are confident that the manuscript is scientifically acceptable.

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The choice of cropping a blot was not done in the same way for all experiments. This was most evident for the protein CEP78 that often exhibited 2 bands with only one of them being specific. In some cases, the non-specific band is shown while absent in others. This does not alter the conclusions, but we recommended consistency in presenting these data with a preference toward including the non-specific band if they are re-occurring, for transparency purposes. Dr. Tsang agreed with our suggestions.

Further, Dr. Tsang brought to our attention that most of the new data was generated from scratch. He also acknowledged that, in a few cases, samples were re-run on new gels when he was sure that they were labelled correctly. If requested, he could indicate which data is completely new and which one result from a re-run of a previous experiment. We asked Dr. Tsang about the significant amount of data that was lost but included in the original submission. Apparently, in addition to lost data, there are also many blots in the student archives that are not labelled correctly such that Dr. Tsang decided to consider this data unusable and prefers to label it as “lost”. However, we remain convinced that the current paper is scientifically sound.

Final conclusion:
We conclude that the data presented in the manuscript resubmitted at EMBO Reports is authentic and scientifically correct, as far as we could determine.

3rd Editorial Decision 03 January 2017

Thank you for the submission of your revised research manuscript to EMBO reports. We have now received the results of the internal investigation on your manuscript and as the committee notes that the data presented in the manuscript resubmitted to us is authentic and scientifically correct, we will proceed with your manuscript. However, before formal acceptance, there are some editorial requests that need to be addressed in a final revision.

Please provide all figures in high resolution (300 dpi or higher) and correct format. See: http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Regarding the source data, please separate the source data and submit one PDF file per figure (including western and IF images). Please also here provide all figures in high resolution (300 dpi or higher).

Further, please orient the western blots in the source data files all in the same way, with the high molecular weight up and the lowest down in the panels (e.g. turn the blot for actin in Fig. 1A accordingly - there are several other cases like this).

For Fig. 2B the panel for Cullin4A-IP flag does not match with the source data (where a band can be seen). Please correct this!

For Fig. EV1D the box in the source data file indicating the shown panel for Cep78 is much smaller than what is in the figure. Please correct this!

In Fig. S3C and D the loading control is labeled alpha-tubulin, in the source data there is written alpha-actin. What is correct?

Finally, the committee stated: "The choice of cropping a blot was not done in the same way for all experiments. This was most evident for the protein CEP78 that often exhibited 2 bands with only one of them being specific. In some cases, the non-specific band is shown while absent in others. This does not alter the conclusions, but we recommended consistency in presenting these data with a preference toward including the non-specific band if they are re-occurring, for transparency purposes. Dr. Tsang agreed with our suggestions." We therefore ask you to do this for all Western panels it applies to in the final revised version of the manuscript.
I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision - authors' response 13 January 2017

Thank you for proceeding with our manuscript and for the opportunity to address your requests. Below please find our point-to-point response.

1) Please provide all figures in high resolution (300 dpi or higher) and correct format.

-We have now provided our figures in 300dpi resolution and correct format.

2) Regarding the source data, please separate the source data and submit one PDF file per figure (including western and IF images). Please also here provide all figures in high resolution (300 dpi or higher).

-We have separated the source data as requested and they are also in 300 dpi resolution.

3) Further, please orient the western blots in the source data files all in the same way, with the high molecular weight up and the lowest down in the panels (e.g. turn the blot for actin in Fig. 1A accordingly - there are several other cases like this).

-We have oriented the western blots with the high molecular weight up and lowest down for Figures 1A (beta-actin), 4C (IN: Flag for MG132) 4D (IN: beta-actin), Appendix Figure S2E (IP: Myc, WB: Myc; IN: Cep78 and IN: beta-actin), Appendix Figure S4A (beta-actin) and Appendix Figure S4B (beta-actin).

4) For Fig. 2B the panel for Cullin4A-IP flag does not match with the source data (where a band can be seen). Please correct this!

-We apologize for the error. The band was supposed to be non-specific since it ran at a much lower molecular weight than cullin 4A (88 kDa). As a consequence, the red box on the right should have been shifted upward. This is indeed what we had in the previous version (July 29, 2016) since this blot had not been replaced in the current version.

5) For Fig. EV1D the box in the source data file indicating the shown panel for Cep78 is much smaller than what is in the figure. Please correct this!

-We again apologize for the oversight. We have corrected the mistake. We have also adjusted the box in the source data for Appendix Figure S2B (IP: Flag, WB: HA) as it was a bit too big in the previous version.

6) Why are sometimes bands for two different proteins on the same source data blot (e.g. Fig. EV2C DDB1 and Cep78)?

-For Fig. EV2C, the blot was first probed with DDB1, followed by Cep78 without stripping.
In another example (Fig. 2B), the blot was cut into two. The top part was probed with EDD1 while the bottom part was probed with Cullin4A.

7) In Fig. S3C and D the loading control is labeled alpha-tubulin, in the source data there is written alpha-actin. What is correct?

-We have now corrected the mistake. It should be alpha-tubulin.

Furthermore we have noticed and corrected several minor errors:
-Figures 6G and I (it should be GT335 instead of GT355)
-Figure 7B (IN: DYRK2 on the source data was not labelled in the previous version)
-Figure EV3B (it should be IP: Flag; WB: VprBP instead of IN: Flag; WB: VprBP on the source data)
-Appendix Figure S1D (a box was added on the source data for IP: Flag; WB: HA)
-Appendix Figure S3A; left blot (IN: Flag should be IN: CP110; IP: Flag, WB: Flag should be IP:

Flag; WB: CP110 on the source data)
-Appendix Figure S5B (IN: Myc-VprBP on the source data was not labelled in the previous version)
-We have also adjusted the molecular weight marker on several blots due to slight inconsistencies between the figures and the source data.
Figures 4D (IP: Flag, WB: HA)
Figure EV5E (IP: Flag, WB: HA)
Appendix Figure S2B (IP: Flag, WB: HA)
Appendix Figure S2D (IP: Flag, WB: HA)
Appendix Figure S2 E (IP: Myc, WB: HA)

8) Finally, the committee stated: "The choice of cropping a blot was not done in the same way for all experiments. This was most evident for the protein CEP78 that often exhibited 2 bands with only one of them being specific. In some cases, the non-specific band is shown while absent in others. This does not alter the conclusions, but we recommended consistency in presenting these data with a preference toward including the non-specific band if they are re-occurring, for transparency purposes. Dr. Tsang agreed with our suggestions." We therefore ask you to do this for all Western panels it applies to in the final revised version of the manuscript.

-For western blots of Cep78 that clearly exhibit 2 bands (with the bottom band being non-specific), we have now shown both bands. This applies to Figure 1F.

We thank you for your consideration and look forward to receiving your response.

Editorial Decision 03 February 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: William Tsang

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2016-43631V1

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	2-5 independent experiements were conducted for all experiments described.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	For immunofluroescence experiments, at least 75 cells in each condition were scored blindly by at least 2 persons.
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Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

Question	Answer
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).	Antibody information is provided in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Information is provided in the Materials and Methods section.

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Question	Answer
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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

Question	Answer
11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	NA
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).	NA
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).	NA
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22. 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.	NA

G- Dual use research of concern

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