Tetrahymena Poc5 is a transient basal body component that is important for basal body maturation

Westley Heydeck, Brian A. Bayless, Alexander J. Stemm-Wolf, Eileen T. O'Toole, Amy S. Fabritius, Courtney Ozzello, Marina Nguyen and Mark Winey
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MS TITLE: *Tetrahymena* Poc5 is a transient basal body component that is important for basal body maturation

AUTHORS: Westley Heydeck, Brian A. Bayless, Alexander J. Stemm-Wolf, Eileen T. O'Toole, Courtney Ozzello, Marina Nguyen, and Mark Winey
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Heydeck, Winey and colleagues here explore the roles of the centrin-binding protein, Poc5 and its presumptive paralog Sfr1, in Tetrahymena basal body regulation and ciliogenesis. They demonstrate that Poc5 is associated with non-mature/assembling BBs and that it is lost prior to ciliogenesis. They find that TtPoc5 causes an increase in cortical BB density accompanied by a decline in ciliation frequency. The authors then demonstrate a functional redundancy between Poc5 and Sfr1 in BB regulation and ciliogenesis. Double knockouts show markedly increased BB density and impaired ciliation, with defective assembly of the ciliary transition zone revealed by electron microscopy. These findings provide new information on the roles of Poc5 in regulating the distal end of the centriole/basal body and in controlling ciliogenesis.

Comments for the author

The data are robust and clearly presented. The principal conclusions are supported by the data. However, given the insights already published by the authors on Sfr1 and its impacts on BB density, it would be useful to provide some additional depth to this study. It would be of particular interest to explore whether the ciliogenesis defect in Poc5-deficient cells stems from an impact on centrin (presumably at the distal end of the BB).

Major points
1. The authors discuss the relationship between Poc5 and centrins in the regulation of centriole/ BB number. It would be useful to present some further characterisation of the BBs overproduced in the Poc5/Sfr1 mutants, particularly with respect to centrin levels. Does Poc5 (and/or Sfr1) deficiency lead to altered composition of the mis-assembling BBs? Are they longer or shorter than controls?

2. Is the ciliary phenotype of the Poc5 knockouts seen in the Sfr1 knockouts (where the authors have previously described a similar phenotype of BB density)? Does a ciliary defect, which the authors propose to drive the compensatory increase in BB density, also arise in these cells?

3. Some quantitation should be provided to support the data on Poc5 behaviour under different growth conditions shown in Figure 3.

4. The BBs in the mCherry-Poc5 experiment in Figure S2 appear different to those seen with the knocked in tag. Are there changes in the BB dimensions with Poc5 overexpression that might relate to overelongation of centrioles with excessive Poc5?

5. A control for the (over-) expression of GFP-Poc5 in the rescued cells used in Figure 4 should be presented. Does the GFP-Poc5 overexpression cause the same impact as the mCherry-tagged form?

Minor points
6. Description of the knockout strategy and its controls should be improved: Figure 4a should indicate the homology arms to scale (or indicating their length) and the PCR primers should be indicated. Formally, the detection of the coNEO cassette does not indicate targeting of the locus (and only the specific region of the targeted locus) without a primer in the 5' or 3' UTR regions. Similarly, the absence of the wild-type signal with no positive control for the PCR is not definitive. In Figure S1, the RT-PCR strategy should be indicated and a positive control provided. The strong background band for the coNEO2 amplification seen only in the poc5 knockout lanes is confusing. The Sfr1 strategy should be included in Figure S3 as well (one presumes it is the same as in the Heydeck paper cited in the manuscript, but it would be helpful to confirm this in the relevant supplementary Figure).

7. All gels should indicate and define the size markers.

8. It should be indicated how often the phenotypes described were seen in the EM experiments.

Reviewer 2

Advance summary and potential significance to field

The manuscript (JOCES/2019/240838) titled “Tetrahymena Poc5 is a transient basal body component that is important for basal body maturation” by Heydeck et al. aimed to characterise Poc5 basal body (BB) functions using the BB-rich Tetrahymena as a model system. The authors began by first identifying a Poc5 homologue in Tetrahymena, TtPoc5, through evolutionarily conserved sequence motif and the organisation of these centrin binding repeats, further validated by the presence of a highly conserved Poc5 box unique to the Poc5 family. They then investigated the localisation of TtPoc5 to BBs using endogenously tagged Poc5-GFP, and Poc1-mCherry as a BB-specific marker. The authors found that TtPoc5 localised to only a subset of the cortical row BBs, exclusively to the assembling BBs. They further verified this finding by testing the localisation in different conditions (growth medium, starvation medium) that favoured towards either assembling or mature-rich BBs. To better understand the recruitment timing of TtPoc5 to BBs, the authors studied its incorporation relative to Sas6a (an early assembly component) and Poc1 (a slowly incorporated component which accumulates during late-stage BB maturation). TtPoc5 is recruited after Sas6a-mediated cartwheel formation but precedes that of Poc1. In addition, given that TtPoc5 was not observed in mature BBs, the authors found that the component was removed from mature BBs prior to ciliogenesis. To study the BB functions of TtPoc5, a complete knockout (KO) strain was generated. The authors reported that the TtPoc5 KO strain was viable, showed unaltered growth rates and morphologically normal oriented cortical row BBs. However, a marked increase in cortical row BB density was observed but with reduced cilia, indicating a function for TtPoc5 in cortical row BB generation with an impact on ciliogenesis. While searching for a Poc5 homologue in Tetrahymena, Sfr1 appeared as the second orthologous hit. A previous study on Sfr1 functions shows phenotypic overlap with TtPoc5 indicating a potential genetic redundancy, thus, the authors attempted to generate a TtPoc5 and Sfr1 double KO strain. The double KO strain proved to be inviable but showed some survivability within a window sufficient to investigate the functional consequences. Cortical row BB density was tremendously increased. The authors examined the ultra-structure of these BBs reporting a varying degree of transition zone formation implying that loss of TtPoc5 and Sfr1 resulted in a BB maturation delay or impaired transition zone formation. All in all, the manuscript characterises TtPoc5 to localise to assembling BBs where it functions in maturation and proper transition zone formation for ciliogenesis and loss of TtPoc5 induces a compensatory response by overproducing BBs.

This is indeed the first study which has investigated the functional characterisation of Poc5 in BBs. Existing studies in the field have focused mainly on the role of Poc5 in centrosomes but there are no published findings highlighting the role of Poc5 in BB or cilia. Reported clinical cases of mutations in human Poc5 imply a function for Poc5 in these structures. Thus this study provides meaningful insights into Poc5’s BB function which can be further investigated in future studies.

Comments for the author

The authors have achieved their aim and the reported findings and conclusions are well-supported by their data. I would recommend the manuscript for publication in the Journal of Cell Science with a few minor improvements and questions to be addressed, as follows:

Improvements
1.For the immunofluorescent microscopy images (Figs 2A and 3C) using Poc1-mCherry, the signal appears to be over-saturated such that details of the oral apparatus membranelles are lost.
2.Figure 4B labelling ‘Wild-type’ should instead be labelled Poc5, as that is what you are detecting via PCR
3.Line 327 ‘deletion of TtPOC5 or SFR1’ should be ‘deletion of TtPOC5 and SFR1’ as you are generating a double heterozygous for both genes
4.Lines 325-333 explaining the steps undertaken to verify the double-KO strain can be improved further by providing a little background to ease understanding especially for readers unfamiliar with the Tetrahymena mating system.
5.Figure 5D, it will be nice to have a EM section showing the transition zone and cilium from a WT strain for comparison with the varying degree of phenotypes shown in the figure

Questions
1.The human Poc5 CBR 2-3 tandem repeat is flanked by short coiled-coils on both sides. Is this also conserved in TtPoc5?
2.Localisation studies were conducted with endogenously tagged Poc5-GFP on the C-terminal. Have you tested the localisation with an N-terminal GFP tag? If so, is the localisation same?
3.Have you done EM on the basal bodies of the single poc5-KO strain? If so, is the transition zone phenotype much milder or similar to the double-KO?

First revision

Reviewer 1 Major points:

1. *The authors discuss the relationship between Poc5 and centrins in the regulation of centriole/ BB number. It would be useful to present some further characterisation of the BBs overproduced in the Poc5/Sfr1 mutants, particularly with respect to centrin levels. Does Poc5 (and/or Sfr1) deficiency lead to altered composition of the mis-assembling BBs? Are they longer or shorter than controls?*

This reviewer did comment about the impact of the work. Unlike our previous Sfr1 work, this current work connects the basal body overproduction with an ultrastructural defect in building the basal body distal end. Also, this work highlights the impact on cilia formation as a result of this impaired basal body maturation. This is an important finding with respect to Poc5 as noted by Reviewer 2. The importance of the genes in question is further revealed in the double knockout (DKO) being lethal, indicating that Poc5 function is essential in Tetrahymena, presumably because of its role in basal bodies.

The effects of these mutants on centrin is an entirely different and very challenging study. There are various centrins at the Tetrahymena basal bodies (see cited Stemm-Wolf, 2005) and many other centrin binding partners at the basal body (see cited Stemm-Wolf, 2013). Furthermore, the mutant phenotypes were analyzed using an anti-Cen1 antibody so, based on that, we know at least Cen1 is still at the basal bodies in these mutant strains (including the DKOs). In the absence of a hint of centrin localization or abundance phenotype, we did not pursue this complex line of inquiry.

With regards to basal body dimensions, we did not see clear differences by light microscopy. There are challenges in doing this work by electron microscopy. Thin sections used in EM do not necessarily include the very distal regions of cortical row BBs, preventing us from making these measurements. We do have 10 tomography datasets of the DKO cells, which represents a significant amount of work. Some datasets included longitudinal images of basal bodies that could be reliably measured, but the numbers are small. No significant difference in basal body length has been detected – DKO lacking cilium was an average length of 460nm (n=5) and DKO with cilium was 460 nm (n=3), and these basal body lengths are similar to what is seen in wild type cells (cited Meehl, 2016).

2. *Is the ciliary phenotype of the Poc5 knockouts seen in the Sfr1 knockouts (where the authors have previously described a similar phenotype of BB density)? Does a ciliary defect, which the authors propose to drive the compensatory increase in BB density, also arise in these cells?*

To address this question, Fig. S3 has been added to show ciliary density phenotype in Poc5 and Sfr1 knockouts compared to WT. The work is described in the text by adding the lines: (Suppl. Figure Legend): “Fig. S3. Reduced ciliary density is specific to loss of Poc5 and not Sfr1. Ciliary density quantification (average # cilia/10 µm) for WT, *poc5Δ*, and *sfr1Δ* cells at 30°C reveals a significantly reduced ciliary density in *poc5Δ* cells, but not in *sfr1Δ* cells. *n*=100 total counts per condition. Error bars: SEM. Student’s t-test calculation used to derive *P* values. ***, $P < 0.001$.” Also, there are the additional lines 316-317 (Results): “Notably, despite apparent BB overproduction in both *poc5Δ* and *sfr1Δ* cells, loss of Sfr1 did not lead to the reduced ciliary density (Fig. S3) observed with loss of Poc5 (Fig. 4F, G).” Lines 537-539 were added to the Discussion: “Additionally, loss of Sfr1 does not

alter ciliary density (Fig. S3), suggesting that TtPoc5 and Sfr1 have functional overlap that modulates BB production, but as their differing BB localization patterns indicate, TtPoc5 and Sfr1 do not appear to be functionally redundant." Finally, we supported the work with lines 735-738 (Materials): "Quantification of the ciliary density (average cilia/10 μm) was quantified for WT, *poc5Δ*, *sfr1Δ*, and *poc5Δ rescue* cells at 30°C. Notably, variable ciliary density measurements were observed across experiments, but a reduced ciliary density in *poc5Δ* cells compared with WT cells was a consistent finding."

3. *Some quantitation should be provided to support the data on Poc5 behaviour under different growth conditions shown in Figure 3.*

The quantitation of the actual number of Poc5-GFP+ BBs was not included, because we are concerned that the numbers could be misleading based on the fact that the copy number of the tagged gene (altering the level of BB signal, which is low) varies based on level of drug selection. Instead, the relative dynamics of Poc5 behavior remains consistent regardless of drug selection: during growth there are a subset of BBs that are Poc5-GFP+ (all cells show a varying number of Poc5-GFP+ BBs), during starvation this signal is largely lost (>95% of cells), and when released after starvation this signal increases beyond what is seen in growth alone (all cells show a varying number of Poc5-GFP+ BBs).

4. *The BBs in the mCherry-Poc5 experiment in Figure S2 appear different to those seen with the knocked in tag. Are there changes in the BB dimensions with Poc5 overexpression that might relate to overelongation of centrioles with excessive Poc5?*

We did not perform EM on cells with Poc5 overexpressed. Furthermore, examination of basal body length by EM is very challenging unless there is a very significant change that is visible by super-resolution light microscopy. With our light microscopy-based studies, we did not detect Poc5 overexpression leading to overelongated BBs (an opposite phenotype of hPOC5 knockdown in centrioles, which would have motivated an EM study). The discussion of the reviewer's first question highlights how challenging it can be to do an EM study that results in small datasets.

5. *A control for the (over-) expression of GFP-Poc5 in the rescued cells used in Figure 4 should be presented. Does the GFP-Poc5 overexpression cause the same impact as the mCherry-tagged form?*

The GFP-Poc5 rescue construct is integrated into the RPL29 locus and under control of the cadmium- inducible MTT1 promoter. This construct rescues the null allele without induction because the promoter is leaky. We show an image of such a cell (Fig. 4D), and cadmium induction leads to such a level of protein that the vacuoles become so bright as to obscure imaging other cellular structures. So, the construct mentioned by the reviewer is not overexpressed in any experiments in the manuscript, and we are unable to determine whether its overexpression produces the structures seen with overexpression of the mCherry-tagged form in a WT background (Fig. S2). We focused on the use of GFP-Poc5 construct to rescue the KO because this is a critical experiment.

Reviewer 1 Minor points:

6. *Description of the knockout strategy and its controls should be improved: Figure 4a should indicate the homology arms to scale (or indicating their length) and the PCR primers should be indicated. Formally, the detection of the coNEO cassette does not indicate targeting of the locus (and only the specific region of the targeted locus) without a primer in the 5' or 3' UTR regions. Similarly, the absence of the wild-type signal with no positive control for the PCR is not definitive. In Figure S1, the RT-PCR strategy should be indicated and a positive control provided. The strong background band for the coNEO2 amplification seen only in the poc5 knockout lanes is confusing. The Sfr1 strategy should be included in Figure S3 as well (one presumes it is the same as in the Heydeck paper cited in the manuscript, but it would be helpful to confirm this in the relevant supplementary Figure).*

We appreciate the reviewer's attention to detail and we have made all of the suggested changes. In Figure 4A, we added scale to homology arms and indicated the location of PCR primers with red arrows. The text of in lines 1121-1122 (Figure Legends) was edited to: "The red arrows indicate the

primers used to amplify PCR products specific to WT POC5 and coNEO2." Lines 641-644 (Materials) were edit to: "…*coNEO2* forward (located in the *POC5* 5' *UTR*, upstream of the integrated *coNEO2* cassette): ATTAATAACATTGCTGATGCTTTT and *coNEO2* reverse (located within the *coNEO2* cassette)…" For Sfr1 strategy (now in Fig. S4) added in text: (Fig. S4 Legend): "This PCR strategy was used to confirm both *poc5Δ* and *sfr1Δ* cells (Heydeck et al., 2016)."

The description of the DKO strategy has been expanded in Lines 323-327 to say: "Briefly, heterokaryon strains with germline deletion of *TtPOC5* or *SFR1* were crossed, and drug-selection followed by PCR confirmation produced heterokaryon cells with germline deletion of both loci (Fig. S4). These cells were then mated together and drug-selected to generate *poc5Δ;sfr1Δ* cells with homozygous deletion of both loci in both nuclei (germline and somatic)."

RT-PCR secondary validation of the poc5 knockout strain (Fig. S1) was further optimized to clean up non-specific bands for amplification of *POC5*, supporting PCR amplification from genomic DNA. This shows clearer amplification of *POC5* in WT cells and not in *poc5Δ* cells. Despite multiple attempts, we were unable to further optimize the *coNEO2* RT-PCR, but only the *poc5Δ* strain (and not WT) is resistant to paromomycin conferred by the neomycin cassette.

7. *All gels should indicate define the size markers.*

This has been addressed. For Figure 4B, "M" was added to indicate markers and we highlighted 0.5, 1.0, 2.0, and 3.0 kbp bands. We also added to text in lines 1124-1125 (Figure Legends): "Sizes of select marker (M) bands are displayed in kilobase pairs."

8. *It should be indicated how often the phenotypes described were seen in the EM experiments.*

To address this question, 10 tomography sets were carefully examined for the defective TZ and ciliary assembly seen in the DKO strain and 7/10 tomograms had at least one basal body affected (some had 2-3 basal bodies affected). There were also normal basal bodies. Three tomograms do not have enough distal region of the basal body in the volume for assessment.

Reviewer 2 Improvements:

1. For the immunofluorescent microscopy images (Figs. 2A and 3C) using Poc1-mCherry, the signal appears to be over-saturated such that details of the oral apparatus membranelles are lost.

The oral apparatus signal is saturated because we did not focus on details of the oral apparatus membranelles and this level of signal is required to interrogate cortical row basal bodies.

2. Figure 4B labelling 'Wild-type' should instead be labelled Poc5 as that is what you are detecting via PCR

We have replaced "Wild-type" with "POC5."

3. Line 327 'deletion of TtPOC5 or SFR1' should be 'deletion of TtPOC5 and SFR1' as you are generating a double heterozygous for both genes

We have restructured the entire section describing double knockout strategy in Lines 321-334.

4. Lines 325-333 explaining the steps undertaken to verify the double-KO strain can be improved further by providing a little background to ease understanding especially for readers unfamiliar with the Tetrahymena mating system.

We have edited lines 323-327: "Briefly, heterokaryon strains with germline deletion of *TtPOC5* or *SFR1* were crossed, and drug-selection followed by PCR confirmation produced heterokaryon cells with germline deletion of both loci (Fig. S4). These cells were then mated together and drug-selected to generate *poc5Δ;sfr1Δ* cells with homozygous deletion of both loci in both nuclei (germline and somatic)."

5. *Figure 5D, it will be nice to have a EM section showing the transition zone and cilium from a WT strain for comparison with the varying degree of phenotypes shown in the figure.*

We have added text to point readers to good examples - line 370 (Results): "(WT comparison found in (Giddings et al., 2010))". Added reference – PMID: 20869521. The figures in the manuscript include our single serial, montage of a longitudinal view of a WT cortical row BB. Fig. S5 (previously S4) does show comparison of WT and double KO BBs at the distal end with cross section images.

Reviewer 2 Questions:

1. The human Poc5 CBR 2-3 tandem repeat is flanked by short coiled-coils on both sides. Is this also conserved in TtPoc5?

We have modified Figure 1A with two green boxes in the schematics of TtPoc5 and hPOC5 to indicate the conserved predicted coiled-coil domains. We added text discussing the coiled-coil domains. This text includes: Lines 168-169 (Results): "as well as predicted coiled-coil domains on both sides of the tandem CBR repeats (green regions in Fig. 1A)…" Lines 578-579 (Materials): "Detection of predicted coiled-coil domains in TtPoc5 and hPOC5 was through the DeepCoil tool (Ludwiczak et al., 2019)." and Line 1074 (Figure Legends): included "….predicted coiled-coil domains (green regions)…"

2. Localisation studies were conducted with endogenously tagged Poc5-GFP on the C-terminal. Have you tested the localisation with an N-terminal GFP tag? If so, is the localisation same?

We have not generated an N-terminally tagged endogenous allele. The rescue construct (exogenous expression) is N-terminally tagged with GFP and it localizes to basal bodies.

3. Have you done EM on the basal bodies of the single poc5-KO strain? If so, is the transition zone phenotype much milder or similar to the double-KO?

We only have EM data from DKO cells so we cannot speak to whether there is a transition zone phenotype in the single KOs. Unlike *Chlamydomonas*, the transition zone in *Tetrahymena* is not well organized making defective structures difficult to detect, therefore we concentrated on the double mutant strain with the most significant phenotype.

Again, our thanks to you and to the reviewers for the efforts on this manuscript. We hope that we have sufficiently addressed the reviewer's comments to make it possible to publish our work in the Journal of Cell Science. Please let us know if you have any questions.

Mark Winey, Ph.D., Distinguished Professor Molecular and Cellular Biology

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Second decision letter

MS ID#: JOCES/2019/240838

MS TITLE: Tetrahymena Poc5 is a transient basal body component that is important for basal body maturation

AUTHORS: Westley Heydeck, Brian A. Bayless, Alexander J. Stemm-Wolf, Eileen T. O'Toole, Amy Fabritius, Courtney Ozzello, Marina Nguyen, and MARK WINEY
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The revision of the paper from Heydeck, Winey and colleagues has addressed the points that I raised in my previous review of the submission. Their paper now presents new information on the functioning of the centrin-binding Poc5 protein in the control of Tetrahymena basal body assembly and ciliogenesis. This study is clearly and convincingly presented and will be of interest to scientists working on the assembly of centrioles/basal bodies and on the regulation of ciliogenesis.

Comments for the author

I have two additional minor comments which might be addressed in the final version of this paper.

1. I suggest that Fig S5 be incorporated into Figure 5. Fig. S5 presents a distinct phenotype and compares the WT and the DKO samples; I think it would be a useful inclusion into the main body of the paper.
2. For completeness, Figs. S2 and S3 should include size markers and Fig. S4 the marker sizes.

Reviewer 2

Advance summary and potential significance to field

This is indeed the first study which has investigated the functional characterisation of Poc5 in BBs. Existing studies in the field have focused mainly on the role of Poc5 in centrosomes, but there are no published findings highlighting the role of Poc5 in BB or cilia. Reported clinal cases of mutations in human Poc5 imply a function for Poc5 in these structures. Thus, this study provides meaningful insights into Poc5's BB function which can be further investigated in future studies.

Comments for the author

The authors have addressed most of my suggestions and questions. I do not have any further comments.