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CryoET shows cofilactin filaments inside the microtubule lumen

Camilla Ventura Santos, Stephen Rogers, and Andrew Carter
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (August 2nd). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

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Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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8) We request authors to consider both actual and perceived competing interests. Please review the new policy () and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any questions or comments regarding the revision.

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

In their paper "CryoET shows cofilactin filaments inside the microtubule lumen", Santos, Rogers, and Carter perform cellular cryo-electron tomogoraphy studies (cryo-ET) that demonstrate the presence of cofilin-satured actin filaments ("cofilactin") within the lumen of microtubules in Drosophila S2 cells treated with the F-actin inhibitor cytochalaisin D. This work significantly expands on a previous study which found distorted actin filaments (F-actin) in the microtubules of human cells (Paul et al., JCB, 2020) treated with a kinesin inhibitor, demonstrating that this phenomenon isn't restricted to a single organism or chemical treatment, but may exist broadly in nature. Furthermore, the authors confidently identify cofilin saturation binding as the cause of the distortion using both subtomogram averaging and docking analysis, as well as RNAi knock-down of cofilin, which results in bare F-actin inside microtubules. They also utilize thapsigargin to increase the prevalence of luminal cofilactin, which they confirm reduces cofilin phosphorylation, consistent with cofilin phosphorylation inhibiting its F-actin engagement inside the microtubule, as expected from its well-established inhibitory role.

Overall, we found this to be clear, concise, and thorough study. It generalizes the existence of intra-luminal actin filaments from one observation in chemically manipulated human cells to a different species, and furthermore characterizes it much more extensively and convincingly. We think this paper will no doubt spur the wider community to look for microtubules featuring luminal F-actin / cofilactin in other species and cell types using cryo-ET and other methods, potentially in more physiological settings where they are likely to be rarer and thus overlooked. Moreover, we appreciate that the authors did not make mechanistic or functional claims beyond the scope of their observations. Consistent with the scope of an EMBO Reports paper, they describe a single striking finding, which is well-supported using a combination of cryo-ET and cell biological manipulations that will serve as a very good example of the rigorous combination of these methods for the field.

We have a few modest suggestions for improving the paper, after which we enthusiastically recommend acceptance by EMBO Reports.

Minor point / suggestion:

The authors report that the registers of tubulin and cofilactin subunits are not correlated, as each becomes smeared out when the other drives alignment during subtomogram averaging. Although this doesn't support a robust cross-talk between the two filaments, they also note hints of possible connecting density, suggesting there may be some binding interactions. We suggest

the authors analyze whether there is a correlation between the polarity of a microtubule and its internal F-actin / cofilactin filament (information they likely already have from their subtomogram averaging analysis). Either a correlation or a lack thereof would be interesting when considering how F-actin internalization might occur.

Small details:
1. Figure 3 A,B show a tomogram slice and crossover lengths obtained from cytoplasmic F-actin. Was this actin filament from these datasets?
2. Figure EV2 D. The legend misstates that the fourth filament is "not clear in this this tomographic slice", rather than the third.
3. It would be nice to include some supplementary movies of the tomograms if allowed by the journal, so that non-specialist authors could appreciate the nature of the data.
4. Kudos to the authors for depositing representative tomograms.

Referee #2:

It was found that cytoplasmic microtubules can contain small proteins or filaments within their structures. Nevertheless, the identity of these proteins and the factors contributing to their accumulation remain uncertain. Santos et al.'s manuscript offers highly intriguing cryo-ET structures of cofilactin in the microtubule lumen, elucidating this previously elusive area of research. The authors convincingly demonstrate that inhibiting the SERCA with thapsigargin increases the frequency of these filaments. Their findings significantly advance our understanding of microtubule-associated proteins within the microtubule lumen. The work is technically robust, supported by a variety of complementary experimental approaches and thorough quantitative analyses. Furthermore, the research topic is highly relevant to the scope of this journal. I have no concerns regarding the main conclusions or the potential impacts of this study in the cytoskeletal field, and thus, I highly recommend its publication after addressing a few minor technical issues.
1. The authors discovered at least 38 protrusions containing microtubules with mixed orientations. This is particularly interesting because a cellular-level mechanism must be involved to regulate the distribution of MTOC in the induced protrusions. I suggest that the authors elaborate on the differences among the various protrusions, explain why some protrusions may contain mixed microtubules while others do not, and propose potential factors that might control the orientation distributions.

2. In relation to the above point, I have several additional questions about the cofilactin structures they determined: What about the orientation distribution of the luminal cofilactin from the reconstructed tomograms? Are the orientations of luminal cofilactin strictly correlated with the MT orientation? What are their orientation distributions in the 3D tomograms? Did the authors observe any preferred locations (outer regions, central regions, or no preference)? What about the orientation distribution changes after SERCA inhibition? Are there any interesting patterns that could potentially be linked to local protrusion geometry and structures? Could these differences subsequently control or affect protrusion growth, cellular cargo transport, and local structural differentiation? The authors do not need to address all these questions in the revised manuscript, but I am curious if there is a possibility to perform slightly more in-depth analyses at the level of whole tomograms to identify any interesting patterns.

3. Given that microtubule protofilaments are nearly straight and parallel to the longitudinal axis, there is a high cross-correlation between a correctly aligned microtubule and misaligned ones in the opposite direction. At the reported resolution, it is possible that certain sub-volumes of the microtubule may not be correctly oriented. The authors clearly demonstrated that protofilaments of microtubules viewed from the plus end appear to rotate in an anti-clockwise direction and conversely in a clockwise direction when viewed from the minus end. However, this is the final result of sub-volume averaging, not the features directly observed from individual sub-volumes of the originally reconstructed tomograms. Because it is an average, even a few misaligned microtubule sub-volumes would not affect the final appearance of this feature at such resolution. Therefore, I am asking whether the authors could provide a validation test on the orientation assignment of a representative microtubule (of average quality) and demonstrate how robust the current alignment is. For example, the authors might consider rotating a set of aligned microtubule segments by 180 degrees, re-refining them, and estimating the cross-correlation coefficients at the opposite orientations. If the differences are not statistically significant, I may have some concern about the final distribution in Figure 1E.

4. A minor formatting issue: In the Materials and Methods section, I noticed that all the units of DNA/RNA concentrations, as well as many other types of units, contain a space after the slash, e.g., µg/ ml, Å/ pixel. It seems more common to use "µg/mL" (without a space before "mL" and many other units), Å/pixel, v/v, etc.

Referee #3:

Please find below the review of the article EMBOR-2023-57264V1 entitled "CryoET shows cofilactin filaments inside the

microtubule lumen" by Santos and co-authors.

In this manuscript, the authors report that in Drosophilia S2 cells, they found in some cases actin filaments (decorated with ADF/cofilin) inside the microtubule lumen. The observation of actin filaments inside the lumen of microtubules has been reported previously Paul et al, JCB, 2020. The originality here is that the actin filaments inside the lumen appear to be decorated by ADF/cofilin.

Decreasing the cellular concentration of cofilin using SiRNA reduces the number of actin filaments inside the microtubule lumen. Overall, the data are very interesting, but the role of this interaction is unclear.
As such, the manuscript describes a curiosity.

Perhaps the authors could clarify whether cofilin-decorated actin filaments are observed outside the microtubule lumen? Do the microtubules protect these filaments from depolymerization?

An interesting possibility raised by the authors is that this is a mechanism of cofilin sequestration, but this mechanism is not challenged by the experiments.

An interesting experiment, if possible, is to polymerize actin in the presence or absence of excess of cofilin in vitro, then add tubulin and assemble microtubules. Will it be possible to observe actin filaments within the lumen of microtubules only if they are decorated by cofilin? This would demonstrate that actin filaments could somehow serve as a template for microtubule assembly.

Point-by-point response

Referee #1:

In their paper "CryoET shows cofilactin filaments inside the microtubule lumen", Santos, Rogers, and Carter perform cellular cryo-electron tomography studies (cryo-ET) that demonstrate the presence of cofilin-saturated actin filaments ("cofilactin") within the lumen of microtubules in Drosophila S2 cells treated with the F-actin inhibitor cytochalasin D. This work significantly expands on a previous study which found distorted actin filaments (F-actin) in the microtubules of human cells (Paul et al., JCB, 2020) treated with a kinesin inhibitor, demonstrating that this phenomenon isn't restricted to a single organism or chemical treatment, but may exist broadly in nature. Furthermore, the authors confidently identify cofilin saturation binding as the cause of the distortion using both subtomogram averaging and docking analysis, as well as RNAi knock-down of cofilin, which results in bare F-actin inside microtubules. They also utilize thapsigargin to increase the prevalence of luminal cofilactin, which they confirm reduces cofilin phosphorylation, consistent with cofilin phosphorylation inhibiting its F-actin engagement inside the microtubule, as expected from its well-established inhibitory role.

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We have analyzed the orientations of microtubules and luminal filaments relative to each other and incorporated the results in the text (lines 122 – 123) and in Fig. EV3E, F. Briefly, our analysis revealed no correlation between the orientation of the luminal filaments and the surrounding microtubules as 46.8 ± 5.5% of luminal filaments had their plus (barbed) ends pointing in the same direction as the surrounding microtubule's plus end (Fig. EV3F).

Small details:
1. Figure 3 A,B show a tomogram slice and crossover lengths obtained from cytoplasmic F-actin. Was this actin filament from these datasets?

The f-actin filament is also from one of the datasets (dataset 5). We have clarified this in the figure legend (Fig. 3A, B).

2. Figure EV2 D. The legend misstates that the fourth filament is "not clear in this this tomographic slice", rather than the third.

We have corrected the figure legend.

3. It would be nice to include some supplementary movies of the tomograms if allowed by the journal, so that non-specialist authors could appreciate the nature of the data.

We have compiled movies of representative tomograms and uploaded them as expanded view files.

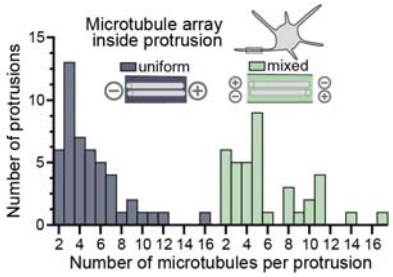
4. Kudos to the authors for depositing representative tomograms.

Referee #2:

It was found that cytoplasmic microtubules can contain small proteins or filaments within their structures. Nevertheless, the identity of these proteins and the factors contributing to their accumulation remain uncertain. Santos et al.'s manuscript offers highly intriguing cryo-ET structures of cofilactin in the microtubule lumen, elucidating this previously elusive area of research. The authors convincingly demonstrate that inhibiting the SERCA with thapsigargin increases the frequency of these filaments. Their findings significantly advance our understanding of microtubule-associated proteins within the microtubule lumen. The work is technically robust, supported by a variety of complementary experimental approaches and thorough quantitative analyses. Furthermore, the research topic is highly relevant to the scope of this journal. I have no concerns regarding the main conclusions or the potential impacts of this study in the cytoskeletal field, and thus, I highly recommend its publication after addressing a few minor technical issues.

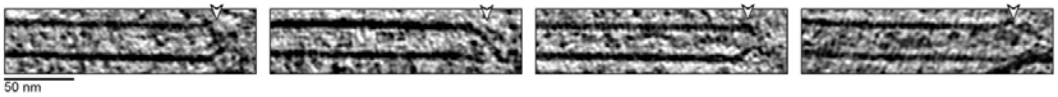
1. The authors discovered at least 38 protrusions containing microtubules with mixed orientations. This is particularly interesting because a cellular-level mechanism must be involved to regulate the distribution of MTOC in the induced protrusions. I suggest that the authors elaborate on the differences among the various protrusions, explain why some protrusions may contain mixed microtubules while others do not, and propose potential factors that might control the orientation distributions.

To test if there were differences between protrusions with uniform or mixed microtubule arrays, we performed three analyses. First, we looked at whether protrusions that contained many or few microtubules tended to have uniform or mixed microtubule arrays. As shown in Referee Figure 1, we observed protrusions with up to 16 or 17 microtubules with uniform or mixed microtubule orientation, respectively, indicating no correlation between the number of microtubules per protrusion and the overall orientations of microtubules therein.



***Referee Figure 1*. Microtubule number inside protrusions with uniform or mixed microtubule arrays.** Histogram of the number of microtubules per protrusion in protrusions with uniform (blue) or mixed (green) microtubule arrays. The cartoon at the top exemplifies uniform/mixed microtubule arrays.

Second, we analyzed whether protrusions with uniform or mixed orientation contained γ-tubulin ring complexes (γ-TuRCs), which could be expected to nucleate microtubules in a particular orientation. Across our data, we observed 89 microtubule ends. Of these, 46 were minus ends and 4 appeared to be capped by structures resembling full or partial γ-TuRCs (Referee Figure 2). These events were too rare to quantify and were present in protrusions with both uniform (n = 2) or mixed (n = 1) microtubules. The presence of γ-TuRCs, therefore, does not seem to determine the overall distribution of microtubule orientations in a protrusion.



Referee Figure 2. Putative γ-TuRCs (arrowheads) observed inside protrusions.

Finally, we looked at whether the distance of the protrusion position from the cell body determined the orientations of microtubules. We estimated the distance of the tomogram acquisition position from the cell body membrane in 47 cases, for which we could confidently trace back the protrusion to the corresponding cell body. Distances were in the range of ~2 – 32 µm. This analysis showed that protrusions with uniform and mixed microtubule arrays were found at similar distances from the cell body.

Overall, our analyses revealed no obvious correlation that would give insights into the mechanism that governs microtubule orientation within protrusions. This is consistent with our prior work showing that known microtubule nucleating factors are not required for steady-state assembly of the acentrosomal microtubule array in S2 cells (Rogers et al. MBOC 19:3163 (2008)). However, research from the Gelfand lab suggested that microtubule-based motors are responsible for generating uniformly oriented microtubule arrays inside protrusions. Based on this, we speculate that microtubules with opposite orientation to the majority within protrusion were captured prior to motor-mediated sorting. As the establishment of microtubule polarity in the cell processes was not the focus of this work, we have included our speculation regarding this mechanism in the manuscript (lines 78 – 80).

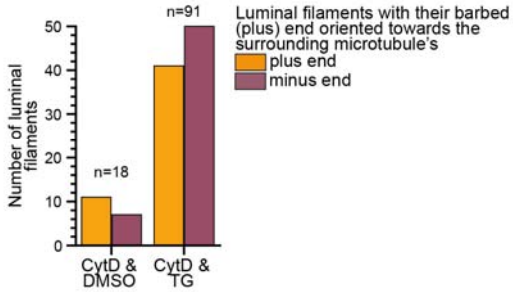
2. In relation to the above point, I have several additional questions about the cofilactin structures they determined:

What about the orientation distribution of the luminal cofilactin from the reconstructed tomograms? Are the orientations of luminal cofilactin strictly correlated with the MT orientation? What are their orientation distributions in the 3D tomograms? Did the authors observe any preferred locations (outer regions, central regions, or no preference)? What about the orientation distribution changes after SERCA inhibition?

As explained in response to Referee #1, we analyzed the orientations of luminal filaments relative to the microtubules within the protrusions and found that 46.8 ± 5.5% of luminal filaments had their plus (barbed) ends pointing in the same direction as the plus end of the surrounding microtubule. We have included two figure panels explaining the quantification and results (Fig. EV3E, F) and incorporated these findings into the text (lines 122 – 123).

Regarding the orientation of luminal filaments within the 3D tomogram, most microtubules, and consequently, luminal filaments, ran along the length of the protrusion.

To analyze changes in luminal filament orientations relative to the surrounding microtubule upon SERCA inhibition, we looked at the number of luminal filaments that were oriented with their plus (barbed) ends towards the microtubule plus and minus end in CytD & DMSO and thapsigargin treated (CytD & TG) cells (Referee Figure 3). Under both treatments, we observed many luminal filaments that were oriented with their barbed (plus) ends towards the microtubule plus and minus ends, indicating no change upon SERCA inhibition.

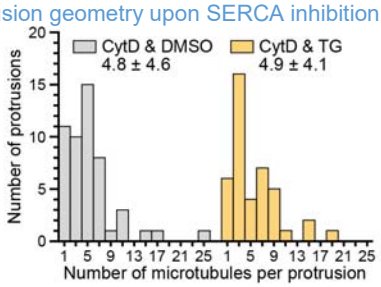


Referee Figure 3. Luminal filament orientation relative to the surrounding microtubule in control and TG-treated cells. Number of luminal filaments in control (CytD & DMSO) or thapsigargin (CytD & TG) treated cells with their barbed (plus) ends pointing towards the plus (n=11, CytD & DMSO, n=41, CytD & TG) or minus (n=7, CytD & DMSO, n=50, CytD & TG) end of the surrounding microtubule.

To look at whether there were preferred locations for the occurrence of luminal filaments (proximal vs. distal regions) we estimated the distance of tomogram positions from the cell body for multiple tomograms with and without luminal filaments. We found no obvious pattern but had too low numbers to approach this in a statistically meaningful way.

Are there any interesting patterns that could potentially be linked to local protrusion geometry and structures? Could these differences subsequently control or affect protrusion growth, cellular cargo transport, and local structural differentiation?

When looking through our tomograms of control and TG-treated cells, we observed no obvious differences in protrusion geometry, which we defined as protrusion width and shape. To gain further insight, we analyzed how many microtubules were contained within each protrusion in control and TG treated cells. This analysis revealed that the targeted protrusions contained very similar numbers of microtubules under both conditions (4.8±4.6 microtubules per protrusion for CytD & DMSO and 4.9±4.1 for CytD & TG, Referee Figure 4). Therefore, we observed no obvious change in protrusion geometry upon SERCA inhibition.



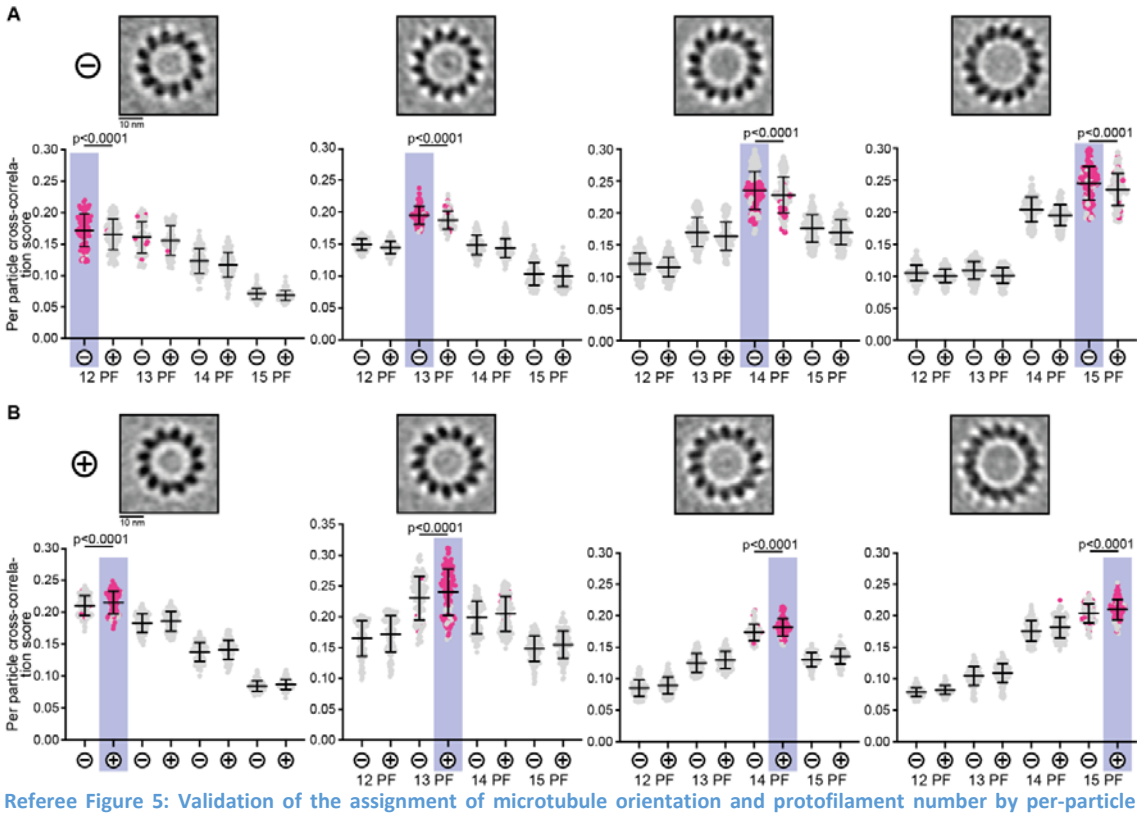
Referee Figure 4. Comparison of microtubule number per protrusion in control and TG-treated cells. Histogram of CytD & DMSO and CytD & TG are shown. Mean ± s.d.s are indicated at the top.

3. Given that microtubule protofilaments are nearly straight and parallel to the longitudinal axis, there is a high

cross-correlation between a correctly aligned microtubule and misaligned ones in the opposite direction. At the reported resolution, it is possible that certain sub-volumes of the microtubule may not be correctly oriented. The authors clearly demonstrated that protofilaments of microtubules viewed from the plus end appear to rotate in an anti-clockwise direction and conversely in a clockwise direction when viewed from the minus end. However, this is the final result of sub-volume averaging, not the features directly observed from individual sub-volumes of the originally reconstructed tomograms. Because it is an average, even a few misaligned microtubule sub-volumes would not affect the final appearance of this feature at such resolution. Therefore, I am asking whether the authors could provide a validation test on the orientation assignment of a representative microtubule (of average quality) and demonstrate how robust the current alignment is. For example, the authors might consider rotating a set of aligned microtubule segments by 180 degrees, re-refining them, and estimating the cross-correlation coefficients at the opposite orientations. If the differences are not statistically significant, I may have some concern about the final distribution in Figure 1E.

The method we used to determine microtubule protofilament numbers and orientations was very similar to that described in our previous publication (Foster et al. 2022). We are now specifically referring to this publication in the manuscript and making clear that the procedures are related (lines 62 – 63 and line 70). In addition, we have expanded Fig. EV1E, F to show the classification result of particles within a representative microtubule. Further representative results and corresponding per-microtubule subtomogram averages of 12 – 15-protofilament microtubules in plus- and minus-end-facing orientations have been included in the Appendix (Appendix Figure S1).

As requested, we have also validated our analysis in two ways. First, we generated per-microtubule averages after the multireference alignment. Visual inspection of these averages gave the same results for the polarity and protofilament number as the assignment by classification. The only exception were microtubules, which were not assigned by either method and whose results could therefore not be compared across methods.
Second, we analyzed the cross-correlation scores. In the multireference alignment used for the polarity determination, each particle is aligned to 8 references (12, 13, 14, 15 protofilament microtubules each with plus- and minus-end-facing orientation). Thus, each particle has a cross-correlation score for comparison to each of the 8 references. We have plotted all cross-correlation scores for particles from 8 representative microtubules (Referee Figure 5). Each particle is assigned to the class (i.e. reference), for which it has the highest cross-correlation score and this value is highlighted in pink for each particle. We performed one-way ANOVA tests with matched values for each particle and multiple comparisons to the assigned class. All ANOVAs and multiple comparisons had p-values <0.0001 and only the significance/p-values between comparisons of classes with opposing orientations (with the same protofilament number) are indicated with bars and p-values in Referee Figure 5.



Referee Figure 5: Validation of the assignment of microtubule orientation and protofilament number by per-particle classification. A, B) Cross-correlation scores for each particle for the alignment to each of the 8 references in the multireference alignment are shown for representative 12, 13, 14, 15 protofilament minus-end- (A) and plus-end-facing (B) microtubules. For each particle, the highest cross-correlation score is shown in pink and this determines the class into which that particle fell. Each microtubule was assigned based on the percentage of particles that fell into each class. The assigned class for each microtubule is highlighted in purple. Per-microtubule subtomogram averages are shown at the top for each of the shown microtubules.

4. A minor formatting issue: In the Materials and Methods section, I noticed that all the units of DNA/RNA concentrations, as well as many other types of units, contain a space after the slash, e.g., µg/ ml, Å/ pixel. It seems more common to use “µg/mL” (without a space before “mL” and many other units), Å/pixel, v/v, etc.

We thank the referee for the comment and adjusted this accordingly.

Referee #3:

Please find below the review of the article EMBOR-2023-57264V1 entitled “CryoET shows cofilactin filaments inside the microtubule lumen” by Santos and co-authors.

In this manuscript, the authors report that in Drosophilia S2 cells, they found in some cases actin filaments (decorated with ADF/cofilin) inside the microtubule lumen. The observation of actin filaments inside the lumen of microtubules has been reported previously Paul et al, JCB, 2020. The originality here is that the actin filaments inside the lumen appear to be decorated by ADF/cofilin.

Decreasing the cellular concentration of cofilin using SiRNA reduces the number of actin filaments inside the microtubule lumen.
Overall, the data are very interesting, but the role of this interaction is unclear.
As such, the manuscript describes a curiosity.

Perhaps the authors could clarify whether cofilin-decorated actin filaments are observed outside the microtubule lumen? Do the microtubules protect these filaments from depolymerization?

We did observe putative cofilactin filaments in the cytoplasm of some protrusions and have added this observation to the manuscript text (lines 153 – 157) with images of these filaments in Fig. EV3I, J.

We have added the speculation that filaments inside the microtubule lumen may be protected from disassembly to the discussion (lines 193 – 194).

An interesting possibility raised by the authors is that this is a mechanism of cofilin sequestration, but this mechanism is not challenged by the experiments.

We have removed the discussion of cofilin sequestration.

An interesting experiment, if possible, is to polymerize actin in the presence or absence of excess of cofilin in vitro, then add tubulin and assemble microtubules. Will it be possible to observe actin filaments within the lumen of microtubules only if they are decorated by cofilin? This would demonstrate that actin filaments could somehow serve as a template for microtubule assembly.

We acknowledge that this experiment would give insights into the prerequisites of luminal filament formation. However, it is not within the scope of the current study. We are aware that another group is actively pursuing these experiments.

Dear Dr. Carter,

Thank you for submitting your revised manuscript to EMBO reports. We have now received the full set of reports from the three referees that were asked to re-evaluate your study. Their comments are included below.

As you will see, all three referees are satisfied with the revision, they mention that the paper has been strengthened and their previous concerns satisfactorily addressed, and they all support publication of your manuscript in EMBO reports.

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Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The authors have thoroughly responded to the reviewer comments, strengthening the already excellent paper substantially. It should be accepted for publication without further delay.

Referee #2:

The authors have carefully addressed my concerns regarding cryo-ET data analysis, and also provided excellent explanations to my additional questions originally just out of curiosity. Thus, I strongly recommend this paper be published.

Referee #3:

The authors have satisfactorily addressed most of my concerns.

The authors have addressed all minor editorial requests.

Dr. Andrew Carter
MRC Laboratory of Molecular Biology
Structural Studies
Francis Crick Avenue
Cambridge Biomedical Campus
Cambridge CB2 0QH
United Kingdom

Dear Dr. Carter,

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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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;
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  - are there adjustments for multiple comparisons?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	The dTAT knock-out S2 cell line is available from the Drosophila Genomics Resource Center (code DGRC#344). This is also specified in the Materials and methods section

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Clone number, catalogue number or citations (for non-commercial antibodies) are specified in the Materials and methods section.

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Sequences are given in the Materials and methods section.

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Used cell lines are specified in the Materials and methods section.
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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Materials and Methods, Figure legends.

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	This information can be found in the Figure legends.
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