JCB

Direct observation of branching MT nucleation in living animal cells.

Vikash Verma and Thomas Maresca

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May 22, 2019

Re: JCB manuscript #201904114

Dr. Thomas Maresca
UMass Amherst
611 N. Pleasantl St.
Amherst, MA 01003

Dear Dr. Maresca,

Thank you for submitting your manuscript entitled "Direct observation of branching MT nucleation in living animal cells". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are impressed by the direct imaging of microtubule branching from augmin in live cells and consider this study appropriate for JCB. Although we do not think it necessary to perform new imaging with a different augmin subunit, a revision should discuss in more detail some of the points raised about evolutionary conservation and the differences with prior work. In addition, there are several requests for further analysis of the existing data to provide more information about, for example, the distribution of augmin along the mother microtubule, and the dwell time and fluorescence intensity of nucleating augmin puncta, that would be interesting and reasonable to include in a resubmission. We also note that the Petry group very recently published a paper on microtubule branching that should be cited and discussed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Tarun Kapoor, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In metazoans, microtubule nucleation is usually seeded by γ-TuRC, facilitating by γ-TuRC attachment factors at different microtubule nucleation centers. One of the microtubule nucleation pathways is mediated by augmin that has been proposed to bind to microtubules within spindle and subsequently recruits γ-TuRC to nucleate new microtubules. The proposed model was largely based on cell biology and biochemical evidence, and how augmin facilitates microtubule branching has never been directly visualized in molecular details. Verma and Maresca applied multi-color TIRF microscopy to study augmin-mediated microtubule nucleation in Drosophila S2 cell line (anaphase). They directly visualized that augmin (Dgt5 subunit) localized at the branching point of microtubules, recruited γ-tubulin and promoted daughter microtubule formation. This observation certainly is an important advance in microtubule nucleation field. However, a deeper investigation using materials they have in hand and methods they have established will be required and will strengthen the paper. Thus, I suggest that the following points should be addressed before publication.

Major concerns:

1. Title: In this manuscript, branch angle they measured in S2 cell is closer to branching microtubule in cortical interphase microtubule arrays in the plant. Furthermore, they also found that Ran and TPX2 that were found to be involved in augmin-mediated microtubule nucleation in Xenopus egg extracts are not needed for augmin-dependent microtubule nucleation in S2 cell line. These findings suggest that the augmin pathway they studied in the anaphase of S2 cell line may not be

evolutionarily conserved in all eukaryotic cells. In the plants, the cell-cycle specific augmin assembly has been proposed as different Hice1/HAUS8 homologs have been shown to mediate microtubule binding of augmin in a cell cycle-dependent manner. Hence, the pathway authors studied may only exist/function in fly and plants. Hence, it is not clear to me why authors used the title: Direct observation of branching MT nucleation in living animal cell. The title needs to be more specific.

2. In addition to imaging Dgt5 subunit of augmin, at least Dgt4, Hice1/HAUS8 homolog, should also be visualized and analyzed so that authors can conclude whether an evolutionarily conserved augmin complex or augmin with different protein composition facilitates microtubule branching in the anaphase they observed.

Authors mentioned that Dgt5 localized as discrete puncta along anaphase microtubules. I think two interesting questions can be addressed in a quantitative manner:

3. Different in vivo and in vitro systems have revealed different dwell time/half-life on the spindle microtubule (minutes) or in vitro polymerized microtubule (seconds). Authors should compare dwell time difference on microtubules for Dgt5 spots that nucleate microtubules and do not nucleate microtubules.

4. What is the intensity difference of Dgt5 spots that nucleate microtubules and do not nucleate microtubules? Augmin is composed of equal stoichiometric subunits based on the information from the biochemical reconstituted augmin complex. However, γ-TuRC contains multiple copies of Nedd1, a component of γ-TuRC and mediates augmin binding. Hence, it's puzzling that whether augmin assembles into a higher order structure and then recruits γ-TuRC for new microtubule formation and spot intensity may help us to answer the question.

5. At the end of the manuscript, the authors showed a correlation between augmin and RhoA pathways and they contribute to furrow ingression and cytokinesis. However, they did not provide a clear biological explanation for this. Can it be connected to microtubule minus movement authors also observed (Page 6, line23).

Minor points:

1. Structures of biochemical reconstituted human and Xenopus augmin both were presented by negative-stain EM approach, not by Cryo-EM (page 10, line14).

2. Cartoon model should be modified and more precise according to the current understanding of γ-TuRC/augmin. Augmin (~400 kDa) is much smaller than γ-TuRC (~2 MDa) and currently we still don't know how many molecules of augmin bind to a γ-TuRC.

Reviewer #2 (Comments to the Authors (Required)):

Verma and Maresca observed, for the first time, augmin-mediated branching-microtubule (MT) generation in live animal cells. They observed anaphase cells of Drosophila S2 cell line using live TIRF microscopy, and co-traced MTs, augmin (Dgt5 subunit), and gamma-tubulin. In majority of the cases, the branching nucleation process began with augmin's binding to existing 'mother' MTs, followed by gamma-tubulin recruitment and new 'daughter' MT nucleation. Remarkably, the angle between mother and daughter MTs was on average 36{degree sign}, which is similar to the angle observed in interphase MT arrays in plant cells and differs from those observed in animal spindles or

axons (nearly parallel, ~0{degree sign}). Another finding is that TPX2, a critical factor for branching nucleation reaction in Xenopus egg extracts, is dispensable in the S2 system. Finally, the larger branching angle nucleation during anaphase spreads out Rho signals for cytokinesis, as new plus-ends of MTs carry Rho activators.

Overall evaluation:
The experiments in this paper were nicely executed and the results are very interesting. I thought visualization of branching MT nucleation in live dividing cells is impossible with the currently available technology since those cells have either dense MT networks (spindles) or highly dynamic MTs (mitotic asters, which would not serve as mothers). The authors' focus on anaphase asters, which are less dynamic than pre-anaphase and reach cortical regions (i.e. TIRF field), was a brilliant idea. The novelty of this finding might be limited if previous plant work is taken into account, where a similar mode of branching nucleation has been reported in plant interphase MT arrays. However, previous reports on spindles (Kamasaki et al., 2013, JCB), axons (Sanchez-Huertas et al., 2016, Nat Commun), and egg extracts (Petry et al., 2013, Cell) suggested parallel MT nucleation operating dominantly in animal cells. Moreover, the current study conducted 3-colour imaging of augmin, g-tubulin, and MTs, convincingly showing the sequential events during branching nucleation and the subsequent MT dynamic instability (plant studies used just two colours). In my opinion, the current discovery is novel enough and would make a nice report in the JCB.

Specific comments:
1. Does a single augmin complex stimulate MT nucleation, like the one sketched in Fig. 5? I believe the authors can provide information on the number of augmin molecules at the branching point based on the fluorescent intensity, as augmin is a hetero-octameric complex. Fig. 2 indicates that not all augmin on MTs recruit gamma-tubulin. Is it possible that only clustered augmin can recruit gamma-tubulin?
2. Please provide control data, where gamma-tubulin and MTs are imaged after augmin depletion. Here, branching nucleation might not be detected at all. Alternatively, different branch angle nucleation might be observed, as suggested in studies involving plant cells (Liu et al., 2014) and axons (Sanchez-Huertas et al., 2016).
3. The authors discuss a possibility that spatial constraints in the environment would affect the branch angle. Could the abundance of MT crosslinkers be another factor? I assume that in the crosslinker-rich region, such as metaphase spindles, widely branched MTs might get crosslinked with other MTs immediately after nucleation and oriented in a parallel fashion.
4. Abstract: I think '16s' and '15s' are mistakenly placed in reverse order.

Reviewer #3 (Comments to the Authors (Required)):

In this work, Verma and Maresca directly visualize branching nucleation in S2 cells using TIRF microscopy. The authors find that augmin precedes gamma tubulin binding which subsequently creates a new microtubule. They also find that TPX2 has no effect in branching nucleation in S2 cells. Microtubule nucleation is one of the most important and less understood processes in assembling microtubule structures. Although there has been extensive evidence of this process (including some recent references that the authors missed and I think they should include, Decker 2018 and Kaye 2018, Oh 2016, where they show that microtubule branching nucleation is the dominant process in Xenopus spindles, and how that nucleation depends on the Ran pathway), the assay presented here allows for characterizing the dynamics of this process which is novel and relevant to the field. I feel the authors could characterize further this process by studying the distribution of augmin binding to the mother microtubule; this would provide very interesting data to

understand how the age of the mother microtubule may affect the branching of new microtubules (David 2018). Finally, the authors speculate on why the branching angle varies so dramatically between what the Petry lab has measured and their measurements. They speculate that the crowding of Mts may affect this angle. They argue that in spindles Mts are very crowded and in growing astral Mts, for instance during interphase (Ishihara 2016), the new microtubules grow in the cytoplasm. This is an interesting speculation, but somewhat difficult to imagine as the main reason, mainly because bipolar spindles can transition to monopoles upon eg5 inhibition in what resembles outgrowth of Mts in aster like structures, similar to interphase asters. If their model would be correct wouldn't one expect that the change in microtubule orientation happens at the boundary of the spindles? I would imagine that the role of molecular motors in keeping Mts parallel is presumably more important. However, it is an interesting idea that needs to be further explored in the future.

Responses to reviewer #1:

Major concerns:

Major Point 1. Title: In this manuscript, branch angle they measured in S2 cell is closer to branching microtubule in cortical interphase microtubule arrays in the plant. Furthermore, they also found that Ran and TPX2 that were found to be involved in augmin-mediated microtubule nucleation in Xenopus egg extracts are not needed for augmin-dependent microtubule nucleation in S2 cell line. These findings suggest that the augmin pathway they studied in the anaphase of S2 cell line may not be evolutionarily conserved in all eukaryotic cells. In the plants, the cell-cycle specific augmin assembly has been proposed as different Hice1/HAUS8 homologs have been shown to mediate microtubule binding of augmin in a cell cycle-dependent manner. Hence, the pathway authors studied may only exist/function in fly and plants. Hence, it is not clear to me why authors used the title: Direct observation of branching MT nucleation in living animal cell. The title needs to be more specific.

Response: The comments on cell-cycle specific regulations and "evolutionarily conserved" aspects of this process are appreciated. In response, we have added language to the text on these topics and comparisons with the plant interphase versus mitotic augmin complexes including a citation of Lee et al. Current Biology 2017 on pp. 6 and 7. However, it is unclear why the points raised in this critique invalidate the title. In fact, one can reasonably argue an opposing point of view; namely that the data presented here, which is fully consistent with that observed in plant cells, does not mean that this pathway only exists/functions in fly and plants (as stated in the comment), but rather that the *in vitro* egg extract system has not adequately recapitulated physiological (and conserved) branching mechanisms that function *in vivo*/in cells. Most of the works from *Xenopus* egg extract refer generally to the process as "branching microtubule nucleation" in their titles without referencing any possible evolutionary conservation or *in vitro* caveats and so we feel it unnecessary to do so here.

Major Point 2. In addition to imaging Dgt5 subunit of augmin, at least Dgt4, Hice1/HAUS8 homolog, should also be visualized and analyzed so that authors can conclude whether an evolutionarily conserved augmin complex or augmin with different protein composition facilitates microtubule branching in the anaphase they observed.

Response: While it may be worthwhile to image Dgt4 in comparison to Dgt5, additional experiments were not deemed to be necessary. Nonetheless, as mentioned in the point above, the important concept of cell-cycle specific augmin complexes with different Hice1 homologues that have been reported in plants have now been added to the text on pp. 6 and 7.

Authors mentioned that Dgt5 localized as discrete puncta along anaphase microtubules. I think two interesting questions can be addressed in a quantitative manner:

Major Point 3. Different in vivo and in vitro systems have revealed different dwell time/half-life on the spindle microtubule (minutes) or in vitro polymerized microtubule (seconds). Authors should compare dwell time difference on microtubules for Dgt5 spots that nucleate microtubules and do not nucleate microtubules.

Response: This is a great suggestion and so we have re-analyzed our data to measure the dwell-time of Dgt5 puncta that support branches versus those that do not. Interestingly, we have found that the Dgt5 dwell time, which includes Dgt5 binding, the duration of the branching event, and Dgt5 dissociation, is longer than the dwell time of puncta that do not support branching events. Specifically, the mean dwell time of Dgt5 puncta that supported branching nucleation was 58.3s ± 24.0s versus 9.9s ± 6.4s for non-branching Dgt5 puncta. The measured dwell times and the 5.9 fold-difference between them are nearly the same as measurements made for nucleating versus non-nucleating augmin and γ-TuRC components from three different studies in plant cells. The new data is shown in Figure 2G.

Major Point 4. What is the intensity difference of Dgt5 spots that nucleate microtubules and do not nucleate microtubules? Augmin is composed of equal stoichiometric subunits based on the information from the biochemical reconstituted augmin complex. However, γ-TuRC contains multiple copies of Nedd1, a component of γ-TuRC and mediates augmin binding. Hence, it's puzzling that whether augmin assembles into a higher order structure and then recruits γ-TuRC for new microtubule formation and spot intensity may help us to answer the question.

Response: This is also an excellent point and we think it is a real possibility that multiple augmin complexes are required to efficiently recruit γ-TuRC. Thus, we re-analyzed our data to measure Dg5 puncta intensity. Interestingly, we did not measure a significant difference in the fluorescence intensity between Dgt5 puncta that supported branching versus those that did not. The new data is shown in Figure 2F. We are careful to also note in the text that this data does not preclude the possibility that augmin oligomers recruit γ-TuRC and also highlight that further investigation of this possibility is warranted.

Major Point 5. At the end of the manuscript, the authors showed a correlation between augmin and RhoA pathways and they contribute to furrow ingression and cytokinesis. However, they did not provide a clear biological explanation for this. Can it be connected to microtubule minus movement authors also observed (Page 6, line23).

Response: We apologize for the confusion. This observation is directly related to a pathway that we recently described in an *eLife* paper (Verma and Maresca, 2019) in which we showed that the plus-ends of astral MTs recruit the RhoA GEF ECT2 and activate RhoA upon physically contacting the plasma membrane. Here, we propose that branching MT nucleation acts as a mechanism to amplify this pathway by generating new daughter MTs that contact the plasma membrane and further activate RhoA. The text has been clarified to better describe this point on p. 11.

Minor points:

Minor Point 1. Structures of biochemical reconstituted human and Xenopus augmin both were presented by negative-stain EM approach, not by Cryo-EM (page 10, line14).

Response: Thank you for catching this oversight. We have now corrected the mistake.

Minor Point 2. Cartoon model should be modified and more precise according to the current understanding of γ-TuRC/augmin. Augmin (~400 kDa) is much smaller than γ-TuRC (~2 MDa) and currently we still don't know how many molecules of augmin bind to a γ-TuRC.

Response: The 5-fold difference in MW is certainly noteworthy; however, MW and 3D spatial organizations are not necessarily correlated since protein size and shape can differ significantly even for proteins of comparable MWs. In this case, we were careful to base the cartoon on published EM data from the Kapoor and Petry labs. For example, the length of the augmin "stem" (30 nm) is slightly larger than the diameter of the MT (25 nm) – this is reflected in the schematic. Similarly, the distance between the fully splayed short and long arms of the augmin complex is ~20 nm by EM (Hsia et al., NCB, 2015), which is comparable to the diameter (20-25 nm) of purified γ-TuRC (Song et al., JCB, 2018) and the MT diameter – we did our best to scale this appropriately in our cartoon as well. One change that was made to the cartoon in response to the point about stoichiometry is to draw attention to the fact that we do not know how many augmin complexes bind to γ-TuRC at the branch point so we have made note of this unknown in an updated cartoon.

Responses to reviewer #2:

Point 1. Does a single augmin complex stimulate MT nucleation, like the one sketched in Fig. 5? I believe the authors can provide information on the number of augmin molecules at the branching point

based on the fluorescent intensity, as augmin is a hetero-octameric complex. Fig. 2 indicates that not all augmin on MTs recruit gamma-tubulin. Is it possible that only clustered augmin can recruit gamma-tubulin?

Response: This is a great question and so we have quantified and compared the fluorescence intensity of augmin puncta that support branching versus those that do not support branching. As stated in our response above to a similar suggestion raised by reviewer 1:

"Interestingly, we did not measure a significant difference in the fluorescence intensity between Dgt5 puncta that supported branching versus those that did not. The new data is shown in Figure 2F. We are careful to also note that this data does not preclude the possibility that augmin oligomers recruit γ-TuRC and that also note that further investigation of this possibility is warranted."

Also, we made a minor addition to the schematic in Figure 5 to draw attention to the fact that we do not presently know how many augmin complexes bind to γ-TuRC at the branch point. This will be interesting to investigate in future work.

Point 2. Please provide control data, where gamma-tubulin and MTs are imaged after augmin depletion. Here, branching nucleation might not be detected at all. Alternatively, different branch angle nucleation might be observed, as suggested in studies involving plant cells (Liu et al., 2014) and axons (Sanchez-Huertas et al., 2016).

Response: While it may be worthwhile to investigate whether near parallel branch angles can be observed in augmin depleted cells S2 cells, additional experiments were not deemed to be necessary at this time. We have added a citation to Sanchez-Huertas on p. 12-13.

Point 3. The authors discuss a possibility that spatial constraints in the environment would affect the branch angle. Could the abundance of MT crosslinkers be another factor? I assume that in the crosslinker-rich region, such as metaphase spindles, widely branched MTs might get crosslinked with other MTs immediately after nucleation and oriented in a parallel fashion.

Response: We agree and so new language has been added language on p. 12 to point out that mitotic motors (per reviewer 3's point) and MT cross-linkers are also very likely contributing to branch angles.

Point 4. Abstract: I think '16s' and '15s' are mistakenly placed in reverse order.

Response: Thank you for catching this error, which has now been corrected.

<u>Responses to reviewer #3:</u>

Point 1: …including some recent references that the authors missed and I think they should include, Decker 2018 and Kaye 2018, Oh 2016…

Response: These references as well as another very recent publication by Thawani et al. 2019 have all been added to the manuscript.

Point 2: I feel the authors could characterize further this process by studying the distribution of augmin binding to the mother microtubule; this would provide very interesting data to understand how the age of the mother microtubule may affect the branching of new microtubules (David 2018).

Response: Great suggestion! In addition to the distribution of Dgt5 puncta, we also analyzed the position of bona-fide branching points along mother MTs and found that in both cases (augmin distribution and branch origins) while there was a bias towards the minus-end, the mean position on the mother MT from which a daughter originates is not at the immediate minus-end but rather, on average, ~37% fractional

position on the mother MT from the minus-end (that could be visualized within the TIRF field). Taken together these data suggest that while there is a minus-end bias, which is likely linked to the variation in age over the length of the mother MT (as the MT the minus-end is inherently older than the plus-end), there may also be spatial regulation of branching in cells that prevents branching at the immediate minus-end. The fact that augmin distribution showed a similar distribution as the branch origin distribution – skewed towards the minus end but lower frequency at the immediate minus end as the branch origin distribution suggests that augmin's MT binding affinity may be spatially regulated during anaphase. Although γ-TuRC recruitment by augmin, and/or γ-TuRC's nucleation activity could also be spatially regulated. These data are now reported in Figure 1F and Figure 2E.

Point 3: Finally, the authors speculate on why the branching angle varies so dramatically between what the Petry lab has measured and their measurements. They speculate that the crowding of Mts may affect this angle. They argue that in spindles Mts are very crowded and in growing astral Mts, for instance during interphase (Ishihara 2016), the new microtubules grow in the cytoplasm. This is an interesting speculation, but somewhat difficult to imagine as the main reason, mainly because bipolar spindles can transition to monopoles upon eg5 inhibition in what resembles outgrowth of Mts in aster like structures, similar to interphase asters. If their model would be correct wouldn't one expect that the change in microtubule orientation happens at the boundary of the spindles? I would imagine that the role of molecular motors in keeping Mts parallel is presumably more important. However, it is an interesting idea that needs to be further explored in the future.

Response: Agreed. We feel the spatial constraint model we proposed may contribute to branch angle variation, but other non-mutually exclusive mechanisms very likely contribute. We have added language on p. 12 highlighting that mitotic motors and MT cross-linkers (per reviewer 2's point) are also very likely major contributors to branch angles.

June 20, 2019

RE: JCB Manuscript #201904114R

Dr. Thomas Maresca
UMass Amherst
611 N. Pleasantl St.
Amherst, MA 01003

Dear Dr. Maresca:

Thank you for submitting your revised manuscript entitled "Direct observation of branching MT nucleation in living animal cells". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- please provide a short eTOC blurb
- provide supplementary text as separate, editable .doc or .docx file
- add paragraph after the Materials and Methods section briefly summarizing the online supplementary materials (inc. videos)
- add conflict of interest statement to Acknowledgements section
- add author contributions

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B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Tarun Kapoor, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology
