Terminally differentiated osteoclasts organize centrosomes into large clusters for microtubule nucleation and bone resorption

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E22-03-0098
TITLE: "Terminally differentiated osteoclasts organize centrosomes into large clusters for microtubule nucleation and bone resorption"

Dear Dr. Harrison:

Your manuscript has now been seen by two expert reviewers in the field, who both find the work novel, interesting and appropriate for eventual publication in MBoC. They both raise some questions/concerns that need to be addressed before publication, mainly regarding the specificity of the drugs, and some interpretations of the data. Most of these are straightforward that can be addressed fairly readily, and neither reviewer has requested to re-review after revision. Therefore I am confident that we can address any further revisions at the editorial level. Thank you for submitting your work to MBoC, and I look forward to seeing the eventual publication of this lovely study.

Sincerely,
Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Harrison,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

I thoroughly enjoyed reading and reviewing this paper, and I would certainly recommend its publication in MBoC.

Osteoclasts are multinucleated cells that arise from cell fusion, and their job is to degrade and remodel bone. It seems the field has long believed that these cells carry out this job without any centrosomal microtubule nucleation; however, this paper uses absolutely beautiful imaging approaches (confocal, TEM, SIM) to conclusively demonstrate that not only do all centrosomes from previously fused cells persist, but that they nucleate microtubules efficiently as well.

The paper nicely details how centrosomes cluster in osteoclasts, which is important to enhance centralized MT nucleation. The authors also demonstrate that this clustering process is reliant on dynamic microtubules and is facilitated by the the minus-end directed motors KIFC1 and dynein. Most interestingly, the authors demonstrate that disruption of centrosome clustering impairs F-actin ring formation and possibly bone resorption. This mechanism suggests one reason why osteoclast may have evolved to be multinucleate with extra centrosomes.

Overall, this was an extremely well-written, rigorous, and convincing study that defines an interesting cell biological mechanism. Centrosome clustering is typically only studied in terms of cancer cell mitosis, and so this paper brings a unique and important new perspective. I have only minor comments/suggestions.

1. Are KIFC1 and Dynein acting redundantly? What were to happen if both were inhibited? What other clustering mechanisms may be at play?
2. When do osteoclasts stop fusing (especially in the in vitro system)? Is it possible that two small osteoclasts with pre-existing clustered centrosomes are fusing, generating the large osteoclasts with more peripheral and distinct clusters of centrosomes that are observed?
3. I am surprised that the giant clustered mass of centrosomes is equally efficient at MT nucleation/per centrosome after normalization. One would think there would be less surface area/volume in such a large cluster, and that clustered centrosomes would nucleate less MT/centrosome. Any ideas?
4. How long is KIFC1 expressed in post-mitotic osteoclasts? Is there any data on this? I think of KIFc1 as a mitotic protein, and I wonder if the lack of mitosis would gradually lower KIFC1 protein levels.
5. This is beyond the scope of this manuscript, but is there an easy way to assess clustering/nucleation in osteoclasts directly from bone samples? It would be nice to see persistent of centrosomes/clustering in aged osteoclasts.
6. The different drug treatments have the same effect on F-actin rings, even though clustering disruption is different. Any explanation? Were F-actin rings analyzed in all cells, or just cells with centrosome declustering?

Reviewer #2 (Remarks to the Author):

It is widely believed that the majority of terminally differentiated cells do not have functional centrosome-based microtubule organizing centres (MTOCs). In these cells, nucleation of microtubules occurs instead at non-centrosomal MTOCs (e.g. nuclear envelope, Golgi). In this study, the authors make use of terminally differentiated osteoclasts to study MTOCs. There are very few studies that have previously examined MTOCs in osteoclasts and it has remained unclear whether centrosomal MTOCs are retained in these cells and if so, what could be their function. Using high resolution microscopy, the authors show that functional centrosomes are present throughout osteoclastogenesis and form clusters. These clusters are more efficient in nucleating microtubules compared to single centrosomes. Additionally, using small molecule inhibitors that induce centrosome declustering, the authors demonstrate a correlation between centrosome clustering and the ability of osteoclasts to form F-actin rings; a process that is essential for bone resorption.

The finding that osteoclasts contain functional centrosomes that are clustered in interphase is novel and interesting. Moreover, the authors show that clustering depends on microtubule dynamics, KIFC1 and dynein. These factors are known to be important for centrosome clustering in cancer cells during mitosis, but have not previously been implicated in centrosome clustering during interphase. However, the link between centrosome clustering and F-actin ring formation/bone resorption is merely correlative and not supported by any direct evidence. In general the work is well conducted and will be of interest to the MBoC readership after addressing the comments below.

Comments:
In fig. 6A the authors show a decrease in centrosome clustering following treatment with dynarrestin, CW069 and Griseofulvin. However, the effects of dynarrestin and CW069 on centrosome clustering are very minimal. Therefore, I wonder if the observed effects of these inhibitors on F-actin ring formation and bone resorption (Fig. 7) can be attributed to this reduction in centrosome clustering, or rather stems from additional effects of inhibiting Dynein and KIFC1. Related to this, the authors cannot exclude that any of the used inhibitors may have additional effects that could directly impact F-actin ring formation and bone resorption

independent of centrosome clustering. As such, direct evidence linking centrosome clustering and bone resorption is lacking. I suggest the authors to either perform experiments to directly demonstrate that clustering is important or to modify the text and conclusions to acknowledge that this has not been demonstrated (figures 7 and 8).

Do the authors need to use the abbreviation CAC? In general using unknown abbreviations makes the paper more difficult to read.

The authors show that both centrosomal- and non-centrosomal MTOCs are present in osteoclasts. Can the authors provide evidence to demonstrate that non centrosomal MTOCs do not play a role in bone resorption? Could be helpful to discuss.

Ensure all graphs have statistical analyses

DATE: April 22, 2022

MANUSCRIPT #: #E22-03-0098

Dear Dr. Walczak,

Thank you for the referee's comments and your encouragement to submit a revised manuscript to *Molecular Biology of the Cell.* We found the comments raised by the reviewers to be relevant and constructive. Each individual comment has been addressed below. We hope that you find the modifications we have made to the manuscript sufficiently address these comments to consider the manuscript for publication.

Response to reviewer comments (Reviewer comments in italics).

Reviewer 1 comments:

1. Are KIFC1 and Dynein acting redundantly? What were to happen if both were inhibited? What other clustering mechanisms may be at play?

We actually spent some time before our manuscript submission trying to determine if the motors were redundant by treating cells with both motor inhibitors. Unfortunately, even at low concentrations, the cells would lift during the long time periods required for osteoclastogenesis. We also treated mature osteoclasts with both drugs and cells would again lift leaving too few remaining osteoclasts for analysis. We did not determine whether cell lifting was due to toxicity or anoikis. We agree that the motors may have redundant activities and inhibiting both motors would potentially have a greater phenotype on centrosome declustering but unfortunately we were not able to show this experimentally and therefore did not include this analysis in the paper.

Another, unexplored mechanism for centrosome clustering may involve adhesion proteins. While this is purely hand waving, we speculate that E-cadherin could be involved. Our lab has shown changes in E-cadherin levels in osteoclasts (Fiorino and Harrison, 2016) and a recent, landmark paper has shown that E-cadherin knockdown increases cellular contractility, leading to more centrosome movement and clustering in cancer cells (Rhys *et al.*, 2018). We have now mentioned this future direction of research in the Discussion of the revised manuscript.

2. When do osteoclasts stop fusing (especially in the in vitro system)? Is it possible that two small osteoclasts with pre-existing clustered centrosomes are fusing, generating the large osteoclasts with more peripheral and distinct clusters of centrosomes that are observed?

In our in vitro system, the cells will continue fusing until massive syncytia are formed. Using live cell imaging we saw two larger osteoclasts fuse and their centrosome clusters coalesce but we don't have a large enough sample size to conclude whether this is a general phenomenon. However, the Reviewer's comment is interesting and may explain the peripheral centrosome clusters in larger osteoclasts.

3. I am surprised that the giant clustered mass of centrosomes is equally efficient at MT nucleation/per centrosome after normalization. One would think there would be less surface

area/volume in such a large cluster, and that clustered centrosomes would nucleate less MT/centrosome. Any ideas?

As shown in Figure 4C, the centrosomes in clusters are slightly less efficient then lone centrosomes at nucleating microtubules, although these results were not significant. We agree with the Reviewer that the reduction may be due to surface area differences between single versus aggregated centrosomes. We were careful not to oversell this result given our non-significant findings in the manuscript.

4. How long is KIFC1 expressed in post-mitotic osteoclasts? Is there any data on this? I think of KIFc1 as a mitotic protein, and I wonder if the lack of mitosis would gradually lower KIFC1 protein levels.

This was a very helpful suggestion and we dug into the literature and found a manuscript showing that KIFC1 increases in osteoclasts in an inflammatory disease model (Chen *et al.*, 2020). We also found strong enrichment of KIFC1 in osteoclasts using bioGPS but are unsure how to include this data in the manuscript. Overall, we were very encouraged by these analyses and now have included this supportive manuscript in our Discussion.

5. This is beyond the scope of this manuscript, but is there an easy way to assess clustering/nucleation in osteoclasts directly from bone samples? It would be nice to see persistent of centrosomes/clustering in aged osteoclasts.

The development of tissue clearing techniques has aided to the identification and cursory examination of osteoclasts in bone tissue but I suspect that the centrosomes are too small to be resolved in cleared bone tissue. But this would certainly be a wonderful addition to these analysis if possible in the future.

6. The different drug treatments have the same effect on F-actin rings, even though clustering disruption is different. Any explanation? Were F-actin rings analyzed in all cells, or just cells with centrosome declustering?

Random fields of view were chosen for analysis in our studies so all osteoclasts were examined. The motor inhibitors had the smallest effect on declustering and this may be due to redundancy as the Reviewer pointed out in Comment #1. Confounding effects may also be due to the relative input of non-centrosomal MTOC MTs as mentioned in Comment#3 to Reviewer 2 (below). It is also entirely possible a critical mass of focal MT delivery is essential to elaborate large sealing zones from F-actin rings but that is purely speculation on our part.

Reviewer #2 comments:

1. In fig. 6A the authors show a decrease in centrosome clustering following treatment with dynarrestin, CW069 and Griseofulvin. However, the effects of dynarrestin and CW069 on centrosome clustering are very minimal. Therefore, I wonder if the observed effects of these inhibitors on F-actin ring formation and bone resorption (Fig. 7) can be attributed to this reduction in centrosome clustering, or rather stems from additional effects of inhibiting Dynein

and KIFC1. Related to this, the authors cannot exclude that any of the used inhibitors may have additional effects that could directly impact F-actin ring formation and bone resorption independent of centrosome clustering. As such, direct evidence linking centrosome clustering and bone resorption is lacking. I suggest the authors to either perform experiments to directly demonstrate that clustering is important or to modify the text and conclusions to acknowledge that this has not been demonstrated (figures 7 and 8).

We did originally raise the drug caveats in the Discussion and mentioned that the drugs may be having a centrosome-independent impact on F-actin ring formation but given that this was raised by both Reviewers, we did a better job of stating this more explicitly in the revised manuscript Discussion.

2. Do the authors need to use the abbreviation CAC? In general using unknown abbreviations makes the paper more difficult to read.

We have now removed this abbreviation in the manuscript and figures.

3. The authors show that both centrosomal- and non-centrosomal MTOCs are present in osteoclasts. Can the authors provide evidence to demonstrate that non centrosomal MTOCs do not play a role in bone resorption? Could be helpful to discuss.

No, in fact the Golgi-based ncMTOCs were also implicated in bone resorption in osteoclasts (knockdown of AKAP6 reduced bone resorption) in the study that we cited in the manuscript. This is now emphasized in the Discussion and the cooperating resources may also explain why we did not see complete abolishment of bone resorption when we inhibit centrosome clustering. We have emphasized this point as well in the Discussion.

4. Ensure all graphs have statistical analyses

We checked that statistical analyses was performed for all graphs as relevant. Some of our graphs were showing frequency distributions and consequently did not require statistics (Figure 3C-E, Figure 6E, Figure 7C, Figure S1E)

<u>References for Editor</u>

Chen, M, Pang, DD, and Dai, SM (2020). Expression Profile of Osteoclasts Following the Stimulation With Interleukin-23 in Mice. Archives of Rheumatology 35, 533.

Fiorino, C, and Harrison, RE (2016). E-cadherin is important for cell differentiation during osteoclastogenesis. Bone 86, 106–118.

Rhys, AD et al. (2018). Loss of E-cadherin provides tolerance to centrosome amplification in epithelial cancer cells. Journal of Cell Biology 217, 195–209.

RE: Manuscript #E22-03-0098R
TITLE: "Terminally differentiated osteoclasts organize centrosomes into large clusters for microtubule nucleation and bone resorption"

Dear Dr. Harrison:

Thank you for making the requested changes to the manuscript. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Claire

Sincerely,
Claire Walczak
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Harrison:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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