The ubiquitin ligases Cbl and Cbl-b regulate macrophage growth by controlling CSF-1R import into macropinosomes.

Adam Hoppe, Lu Huang, Natalie Thiex, Jieqiong Lou, Gulzar Ahmad, Wei An, Shalini Low-Nam, Jason Kerkvliet, and Hamid Band

Corresponding author(s): Adam Hoppe, South Dakota State University

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Editor-in-Chief: Matthew Welch

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 #E23-09-0345
TITLE: The ubiquitin ligases Cbl and Cbl-b regulate macrophage growth by controlling CSF-1R import into macropinosomes.

Dear Adam:
Your manuscript "The ubiquitin ligases Cbl and Cbl-b regulate macrophage growth by controlling CSF-1R import into macropinosomes" has been reviewed by two expert referees. Their verbatim comments are appended below. Both reviewers found the topic interesting and the experiments generally well designed and executed. Nevertheless, they raised a number of issues and made several suggestions that will need to be addressed before the manuscript is reconsidered for publication. This will require some experimental work, whether to provide the additional information requested, or to increase the number of replicates in some instances. The revised version will, in all likelihood, need to be re-examined by the original reviewers.
I look forward to receiving the revised version of your interesting paper.
Best regards.

Sincerely,

Sergio Grinstein
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Hoppe,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments 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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Significance statement needs to be structured in three bullet points as per the instructions: https://www.molbiolcell.org/curation-tools

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Thank you for submitting your manuscript to MBoC. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager

MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript, Huang et al show that combined loss of Cbl and Cbl-b affects endocytosis and intracellular traffic of CSF-1R. the DKO slows CSF-1R internalisation, leads to higher AKT signalling and fails to send the receptor to MVB.
Overall, this an interesting paper and most experiments are well done. However, the manuscript has quite a few typos and there are experiments that are not properly replicated. These replicates need to be performed before the paper is acceptable.
Major point:
• Figure 4a/b/g: are there replicates for this? 4e probably needs also a third replicate!
Minor points.
• Abstract: line 50: singling signaling.
• Line 291 the sentence is not finishing "(bright ring-like objects that rapidly,"
• I find the pictures of Figure 2 too small to read
• Figure 2H what is the chase time?
• Line 409 "...does not reduce and indeed my increase..." - remove "my"
• The quality of Figure 5 is too low to read it properly.
• I am not a transcriptomics expert, but what are the statistical settings used for the transcriptomics analyses? How is FDR calculated?
• Line 501: "through" and "though"
• Line 506: replace "only" with "single"
• Line 517: "did had" "had a"
• Line 518: "lead" "led"
• Line 523: remove "in"
• Line 531: important "in"...
• Line 532: "signalging" signaling
• Line 537: "against"
• Figure legend S1: "gnomic" Genomic

Other comments:
• The use of antibodies in binding to potentially ubiquitylated proteins has some potential drawbacks as the lysines that are ubiquitylated are often the most immunogenic areas to which antibodies bind. i.e. antibodies do not bind anymore to ubiquitylated proteins! A nice example for this can be found here: https://pubmed.ncbi.nlm.nih.gov/36281581/ Figure S3b

• I am rather surprised that the pretty huge difference in phenotype is not showing a much higher change in the gene expression between WT and DKO (Figure 5G). would proteomics perhaps be a better measure of differences?

Reviewer #2 (Remarks to the Author):

In this study, Huang et al. elucidated the contribution of Cbl and Cbl-b on the endocytosis and the effects of sub-cellular compartmentalization of CSF-1R signaling in macrophages, using Cbl-/-, Cbl-b-/- and double knockout (DKO) cells. They showed several interesting findings, for example, Hrs and CSF-1R association mediated by Cbl or Cbl-b in the ESCRT recognition system. However, the Reviewer found several short points as follows.

Major Concerns
1) They must show changes in expression of Cbl and Cbl-b in wild-type, Cbl-/-, Cbl-b-/- and double knockout (DKO) cells after treatment with CSF-1.
Cbl-/- macrophages had a delay and partial reduction in ubiquitination relative to WT on Fig.1. Cbl-1R ubiquitination in Cbl-b-/- macrophages was almost indistinguishable from WT. In addition, the complete loss of CSF-1-induced CSF-1R ubiquitination in DKO macrophages. Therefore, they suggested that Cbl and Cbl-b both contribute to CSF-1R ubiquitination, and that Cbl's activity appears particularly important for the early phase of CSF-1-dependent ubiquitination. However, they did not show any data for the expression of Cbl and Cbl-b in wild-type, Cbl-/- and Cbl-b-/- macrophages. Their data cannot exclude the following possibility: Cbl-1R ubiquitination in Cbl-b-/- macrophages was almost indistinguishable from WT, because the expression in Cbl-b in WT macrophages is quite lower, compared with that in Cbl. In addition, CSF-1-induced Cbl expression may be weak in Cbl-b-/- macrophages.

2) RNA-seq experiments (Fig. 5),
Differential expression analysis indicated that 520 genes were upregulated and 280 were downregulated in DKO macrophages when compared with WT controls and a smaller number (200) of total genes were differentially expressed in Cbl-/- and only 28 in Cbl-b-/- relative to WT (Fig. 5). Then, they concluded that Cbl-had stronger influence than Cbl-b in regulating gene expression. The Reviewer wondered if this conclusion was also overinterpretation, due to the similar reasons described in 1).

Minor Concerns
3) There are many ambiguous expressions.
EX) Line 476, While somewhat counterintuitive,....
Line 533, ...had a somewhat stronger ....

4) Fig. 1 F & G
Figure 1F (Western blotting) was not seemed to be matched to the graph pattern (GSF-1R intensity on Figure 1G).

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Overall, this an interesting paper and most experiments are well done. However, the manuscript has quite a few typos and there are experiments that are not properly replicated. These replicates need to be performed before the paper is acceptable.
Major point:
• Figure 4a/b/g: are there replicates for this? 4e probably needs also a third replicate!

Thank you for your excellent comments. We had performed a number of replicates for these western blots but not presented these as they were completed with supernatant harvests at different times following CSF-1 stimulation. To address this important concern, we have averaged the data from common time points across these multiple western blots. In most cases this has allowed us to present graphs consisting of triplicate or quadruplicate results. The one exception is Figure 4b, which only had timepoints consistent with a duplicate trial across all mutants (although we ran many experiments comparing WT and DKO). Given that these mice are not currently being bred, and the frozen bone marrow cells were lost due to a power failure from a Derecho, we could not repeat that experiment in a timely fashion. However, the critical difference between the WT and DKO macrophages is highly reproducible and the duplicate result shown in figure 4b is consistent with our blots comparing the WT and DKO.

Minor points.
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The only statistical stetting for the differential gene expression analysis was the FDR which was set within DESeq2. We have added a reference containing the details. We set the FDR to 0.05, which can be interpreted as ~ 5% of the differential genes will be detected as false positives. This is a 'middle-of-the-road' setting (not overly stringent, not overly loose) typically found in exploratory RNAseq experiments. We have added a statement to line 442

explaining this along with a reference.
• Line 501: "through" and "though"
• Line 506: replace "only" with "single"
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Thank you for pointing these out! We have carefully edited the entire manuscript fixing these and other errors.

Other comments:
• The use of antibodies in binding to potentially ubiquitylated proteins has some potential drawbacks as the lysines that are ubiquitylated are often the most immunogenic areas to which antibodies bind. i.e. antibodies do not bind anymore to ubiquitylated proteins! A nice example for this can be found here: https://pubmed.ncbi.nlm.nih.gov/36281581/ Figure S3b

Thank you for pointing out this reference. This is a good point that we will keep in mind for future studies of ubiquitylation. In our case, our CSF-1R antibody recognizes the extracellular portion of the CSF-1R so, it is unlikely that Ub would have interfered with this blot or subsequent blots. Definitely an important consideration!

• I am rather surprised that the pretty huge difference in phenotype is not showing a much higher change in the gene expression between WT and DKO (Figure 5G). would proteomics perhaps be a better measure of differences?

We agree that lack of a huge difference in transcriptomics is surprising as we too had expected this analysis to reveal substantial changes in the transcriptional program to indicate a clear mechanism defining the hyperproliferative phenotype of DKO macrophages . While there are some suggestive up and downregulated genes it is a small list. Future studies under more limiting growth factor conditions may help reveal more significant differences since Cbl/Cbl-b combined deletion is known to render cells more sensitive to lower levels of growth factors (Naramura M et al. PNAS 2010). We agree that proteomics would likely be an excellent future direction for interrogating the changes to the CSF-1R signaling complex and the role of Cbl and Cbl-b in its regulation.

Reviewer #2 (Remarks to the Author):

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Thank you for the thoughtful consideration of our study. Indeed, not presenting these data was an oversight on our part. We have provided the expression data for Cbl and Cbl-b across the different BMDM genotypes (Figure 5H) and results are presented on lines 450-453. Here, and similar to other transcriptome work being done in the lab, Cbl and Cbl-b appear to have very similar gene expressions. Indeed, there may be a very small degree of compensation for increased Cbl-b expression in the Cbl-/- BMDMs (~15%), but we observed no change in Cbl expression in the Cbl-b-/- BMDMs. Given this similarity of expression and the finding that the major differences in CSF-1 signaling and growth only occur in the Cbl DKO, we believe it highly likely that, at least for CSF-1 signaling, that Cbl and Cbl-b share overlapping regulatory functions. Indeed, a similar Cbl/Cbl-b regulation of proliferation was

recently identified for CD11c+ macrophages/dendritic cells:
https://www.nature.com/articles/s41420-022-00953-2
We have added this to the discussion and added discussion on the relative expression of Cbl and Cbl-b.

Minor Concerns
3) There are many ambiguous expressions.
EX) Line 476, While somewhat counterintuitive,....
Line 533, ...had a somewhat stronger ....

We have extensively edited the manuscript and corrected these. Thank you!

4) Fig. 1 F & G
Figure 1F (Western blotting) was not seemed to be matched to the graph pattern (GSF-1R intensity on Figure 1G).

Thank you for identifying our oversight. Per reviewer 1's comments, we have quantified these data from multiple western blots and present these as a new graph. We have also corrected the error in the original analysis.

RE: Manuscript #E23-09-0345R
TITLE: "The ubiquitin ligases Cbl and Cbl-b regulate macrophage growth by controlling CSF-1R import into macropinosomes."

Dear Adam:

I am pleased to accept your revised manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Sergio Grinstein
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Hoppe:

Congratulations on the acceptance of your manuscript! Thank you for choosing to publish your work in Molecular Biology of the Cell (MBoC).

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