JCB

Reactive oxygen species (ROS) triggers unconventional secretion of antioxidants and Acb1

David Cruz-Garcia, Nathalie Brouwers, Vivek Malhotra, and Amy Curwin

Corresponding Author(s): Amy Curwin, Centre for Genomic Regulation and Vivek Malhotra, CRG, Centre de Regulacio Genomica

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June 11, 2019

Re: JCB manuscript #201905028

Dr. Amy J Curwin
Centre for Genomic Regulation

Dear Dr. Curwin,

Thank you for submitting your manuscript entitled "Reactive oxygen species (ROS) production triggers unconventional secretion of antioxidant enzymes". The manuscript was assessed by three expert reviewers, whose comments are appended to this letter.

You will see that all of the reviewers feel this work is potentially interesting and appropriate for JCB. There are some common points raised across the reviewers, in particular the significant question of physiological relevance. While for a short Report we would not expect a full mechanistic story, establishing that this event is important for survival seems key. We invite you to resubmit after addressing the following essential revisions:

1. Test the physiological relevance by examining fitness of grh1 (or vps23) mutants and/or sod1/trx1/2 mutants

2. At a minimum, cite the relevant literature from mammalian systems as suggested by Reviewer 1, and preferably explore some of the known kinase pathways that might be relevant.

3. Specify the abundance of the ROS-related hits, and also show specificity by examining secretion (or lack thereof) of other abundant cytosolic enzymes (Reviewer 1, point 2; Reviewer 2, point 1)

4. Provide some additional characterization of mitochondrial function/behaviour as suggested by Reviewers 2 and 3. Please use your discretion in determining the specific experiments that would best address these concerns.

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.

GENERAL GUIDELINES:
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.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available.

Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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,

Elizabeth Miller
Monitoring Editor
JCB

Rebecca Alvania
Executive Editor
JCB

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

This manuscript reports that ROS production triggers unconventional secretion of antioxidant enzymes in starved yeast cells. There is great interest in understanding the secretion of SOD as its secretion in an unfolded state is linked to ALS. The authors show here that SOD is active extracellularly, is exported with other antioxidant enzymes, and export is stimulated by reactive oxygen species generation but not H2O2. The work is carried out to a high standard but the following issues would need to be addressed before publication can be recommended.
1. There are numerous papers in the literature that show a role for ROS in glucose stimulated insulin secretion from beta cells and even in unconventional IL1beta secretion from macrophages that are not cited here and need to be (together with an integration of the previous findings with the present story). The existence of those previous studies also relates to the relative novelty of the present story, and suggest ways for the authors to enhance the present story--in beta cells it seems that ROS influences calcium levels and in macrophages, ROS is linked to kinase activation--the present story would be greatly enhanced if the authors checked those processes to see if they

are part of the anti-oxidant secretion process reported. (See PMID:23963575, 17132626.)

2. It is of course interesting that multiple Vps23-dependent secreted products are anti-oxidants. It would be more compelling if the authors also report their relative abundance to overall cellular proteins to provide an index of their enrichment in this pathway.

3. (Minor) the way in which some of the data are presented (fold change +/-) could be presented more clearly--a 5 fold decrease is easier for the reader to process than a ratio of 0.2. Please replot to make this clearer for the reader.

Altogether, the story would be less incremental if the authors took their assay one step further to get at the mechanism by which ROS triggers this secretory process as seen in the papers described in point #1. One more set of assays would go far in terms of the novelty of this story for readers of JCB.

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

This study is a continuation of previous work from the Malhotra and Curwin labs investigating the mechanisms and function of unconventional protein secretion. This work focuses on the adaptation of yeast cells to acute, 2.5 hr shifts into potassium acetate as a means of inducing starvation. Previously, they observed the generation of a new autophagy related membrane bound compartment they called CUPS that mediates the secretion of ~1% of the cytosolic Acb1 (acetyl CoA binding protein 1) and SOD1, among others. The mechanisms and signaling that regulate this process are unclear, so in this work they performed proteomic analysis of secreted proteins during growth, starvation, and in the presence or absence of an ESCRT component Vps23 and GRASP65 orthologue Gsh1, to identify CUPS-dependent secretion.

They identify additional redox related thioredoxins along with SOD1 and Acb1, prompting them to examine the potential role of mitochondrial metabolism and redox control of this pathway. They show that secreted SOD1 remains functional so they propose that this release mechanism is important for survival. Overall, understanding the mechanisms of unconventional protein secretion is certainly very important. However, the study is preliminary in its current form and the links to their previous work and mechanisms are unclear, making the conclusions difficult to understand. The mitochondrial response to starvation is complex, involving signaling processes, quality control, and potentially direct links to the generation of the autophagosome. Ultimately this study identifies a few more secreted proteins that appear to be linked to redox control and metabolism, and that secretion is blocked with a ROS scavenger. I think the potential to link mitochondrial function, dynamics and signaling to their pathway is very high, but this study hasn't provided a significant advance. I have listed my concerns and suggestions below.

1. The authors identified 136 secreted proteins, 25 were specific to starvation and Vps23 with the values of 16 plotted, and blots shown for 3. What is the input loaded for the thioredoxins? Are these also showing ~1% of the total being secreted? Ultimately the pathway is predicted to be functionally protective somehow, but what is the consequence of starvation within Vps23 or Ghs1 mutants where this process is blocked? Fig 1A uses an in gel assay to monitor SOD1 activity, but does it have substrates when released? Can it really be enough to scavenge from this location? There are no quantitative statistics for this data either, so it is difficult to understand the power of this released SOD1.

2. The choice to look at mitochondrial function during the 2.5 hrs in potassium acetate is important, but figure 2 examines a very bare minimum of mitochondrial function. This is not enough to understand what might be happening. There are also no quantifications or statistics shown, nor a time course through the starvation protocol. Examining potentiometric dyes is no substitute for oxygen consumption measurements. Increased potential can be seen for different reasons, a block in complex V, for example can lead to hyperpolarization.
3. The morphological change into an enlarged, rounded mitochondria is not examined in any detail either. Are these in contact with the vacuole? Are they hyperfused or fragmented? Have they disengaged from the Num1 contacts around the cortex? This must be examined in more detail. There is an emerging (and old) literature concerning the integration of mitochondrial dynamics and function during amino acid starvation, TOR activation, entrance into quiescence, sporulation, etc., that is very complex and completely ignored in this study. I am not aware of others looking at mitochondria in potassium acetate starvation, and for this short period - generally it is amino acid starvation and autophagy conditions. How different is this?
4. It is certainly not surprising that the complete uncoupling of mitochondria with DNP blocks the release, and unclear why this was included since they are just killing the cells most likely.
5. Figure 3 shows an analysis of YAP translocation as a readout of ROS, showing no translocation to the nucleus in their starvation conditions. The result is that addition of the ROS scavenger NAC blocked secretion, but this wasn't clearly linked to mitochondrial respiration directly.
6. The authors have shown previously that the CUPS compartment drives secretion, but this was not examined in the context of mitochondria. Does the CUPS form when mitochondrial dynamics/contact sites are altered? Is there any spatial relationship between CUPS and mitochondria? Mitochondria have been shown (controversially I admit; see Yoshimori PMID:23455425) to contribute to the growth of the autophagosome in some conditions. Is it possible that acute metabolic transition may drive mitochondria to promote extracellular protective cues that may rewire signaling? The Prinz lab showed during stationary phase that the vacuole phase partitions at the lipid level (PMID:23836928), then later showed with Nunnari (PMID:28774891) to require mitochondrial contacts mediated by sterol transporters and regulated by the TOR pathway. It has become clear that major metabolic rewiring is initiated in these situations, and mitochondria are central players in launching these changes. However, the current manuscript has just touched the surface in a very peripheral way, leaving the reader without any additional insights into this important process.

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

Cruz-Garcia and colleagues investigated the signal that triggers starvation-induced unconventional secretion by yeast cells. Using unbiased proteomics of yeast cell walls, they identify 10 candidate unconventionally secreted proteins and they confirm this for three of them (Ahp1, Trx1, and Trx2). Based on the enzymatic activities of Ahp1, Trx1, and Trx2 and their roles in modulating oxidative stress in cells, the authors speculate that oxidative stress triggers unconventional secretion as a protective response for the cell wall. In support of this, they report that incubation of cells with DNP, and inhibitor of mitochondrial respiration, and NAC, a free radical scavenger, reduce unconventional secretion. However, they report that H2O2 (0.1 mM), a commonly used oxidation perturbant, does not have an effect on unconventional secretion, leading to the suggestion that one or more specific species of reactive oxygen produced by mitochondria is/are the trigger.
The identification of a signal that initiates unconventional secretion, and a physiological role for unconventional secretion, are important questions in the field. However, the conclusion that intracellular ROS production by mitochondria triggers unconventional secretion requires additional

control experiments to rigorously substantiate it. No evidence is presented in support of the proposal that unconventional secretion of antioxidants is protective.

1. The authors show that ATP depletion by DNP (figure 2), and treatment of cells with NAC (figure 3), decrease secretion of various proteins. It is assumed that NAC acts by scavenging free radicals, but what is the effect of NAC treatment on cellular ATP levels? What is the effect of DNP and NAC treatment on Yap1-YFP nucleus accumulation in starved cells - is nucleus accumulation prevented?

It might also be interesting and helpful to know if unconventional secretion is affected by deletion of the Sod1, Ahp1, Trx1/2 genes (single mutations, multiple mutations)? It might be expected that unconventional secretion is constitutive in these cells (due to elevated radicals), and/or that the amount of exogenous ROS required to trigger unconventional secretion is reduced. This could be tested by measuring secretion of any of the other newly-identified secreted proteins in a triple mutant cell.

2. It is proposed that unconventional secretion confers protection to oxidative stress, but this is not tested. Does eliminating unconventional secretion of antioxidants compromise cell wall integrity or decrease cell survival (or some other measure of extracellular oxidative challenge) upon extracellular oxidative challenge? This could be measured for cells with deletions of Sod1, Ahp1, Trx1/2, or less rigorously, for Grh1 or Vps23 mutants.

3. The data in Figure 3B do not convincingly show localization of Yap1-GFP to nuclei of starved cells. Localization to the nucleus should be confirmed by co-localization with a marker for the nucleus. Is nuclear accumulation observed in 100% of the cells, as shown in the figure?

4. The conclusion (page 7) that "nutrient starvation upon culture of cells in potassium acetate leads to unconventional secretion of a number of enzymes that function, either directly or indirectly, in response to oxidative stress" is somewhat misleading. It's true that expression and/or activity of many of the unconventionally secreted proteins affects cellular redox, but what is the evidence that the other proteins (ie, in addition to Ahp1, Trx1, and Trx2) "respond" to oxidative stress?

5. What does it mean (page 6) that a protein is "growth specific" or "starvation specific"? What does "...secreted specifically in growth..." mean?

December 18, 2019

Dear Tim,

We are pleased to resubmit a revised manuscript entitled, "**Reactive oxygen species (ROS) triggers unconventional secretion of antioxidants and Acb1**" for publication in JCB. The reviewers have made us rethink about a number of issues and we thank them for helping us improve the quality and to deliver a cleaner message.

In sum, the message we want to convey is that starvation induces ROS production and mitochondria is the most likely source of this product. ROS produced triggers the release of antioxidants and proteins like Acb1. The release propensity of the cells is abrogated by treatment with membrane permeant NAC, which sequesters ROS. Cells that lack Grh1 fail to regrow in the absence of NAC compared to wild type cells when shifted from starvation to growth medium. Altogether, these data allow us, for the first time, to hypothesize the functional significance of unconventional protein secretion upon starvation. Interestingly, Acb1 is emerging as an important lipogenic signalling molecule in mammals and therefore, these studies highlight the involvement of ROS in a number of pathways that are controlled by unconventionally secreted proteins. These findings raise many challenging issues. For example, how are these proteins selected for secretion? Does ROS affect their form or the machinery required for their release? How do other cellular compartments, including mitochondria, CUPS and ER participate in this process? Our understanding of unconventional secretion is rather primitive and our new data will help others and us to move this field forward.

In the letter signed by the monitoring editor Dr. Liz Miller, we were asked to address four issues. Two of the issues were huge challenges that required 1) a SILAC based proteome analysis of growing and starving cells, and 2) an assay to evaluate the functional significance of unconventional sequence. We have addressed the reviewers concerns to the best of our abilities and hope that our revised manuscript is suitable for publication.

The issues and our response, in italics, follow.

1. Test the physiological relevance by examining fitness of grh1 (or vps23) mutants and/or sod1/trx1/2 mutants.

Deletion of the antioxidant enzymes themselves will cause a number of cellular responses unrelated to unconventional secretion. We have thus monitored the effect of deletion of Grh1 and Vps23 on the fitness of cells after starvation. Our data reveal that loss of Grh1and Vps23 severely affects the growth propensity of cells when they are shifted from starvation to growth medium, despite the cells being viable, as measured by Calcein AM fluorescence. We have found that treatment with N-acetyl cysteine (NAC), a ROS quencher, abrogates the growth defects in Grh1, and to lesser extent Vps23, depleted cells. The defects of Vps23 deletion are less restored because of its likely involvement in a number of other processes in addition to its role in unconventional protein secretion. This is shown quantitatively in new Figure 4. This supports the involvement of ROS, Grh1 and Vps23 in unconventional protein secretion and reveal show these activities maintain the cell in a form that is necessary for sustenance during starvation for their ability to grow upon return to growth conditions. This experimental data helps in further supporting the challenging issue regarding the significance of unconventional protein secretion.

2. At a minimum, cite the relevant literature from mammalian systems as suggested by Reviewer 1, and preferably explore some of the known kinase pathways that might be relevant.

We appreciate this concern and wish there was a straightforward means to address this problem. We have recently reported the involvement of GRASP55 and GRASP65 (the mammalian orthologs of Grh1) in secretion of IL-1ß by mouse macrophages. Dr. Nickel has shown the involvement of kinases in trafficking of FGF2, which unlike SOD1 and Acb1 is independent of Grh1, and occurs by direct translocation across the plasma membrane. Dr. Rabouille has reported on GRASP dependent Golgi bypass of an integrin in fly embryos. All these processes appear to follow different routes for reasons unclear to those who are working on these issues. IL-1ß secretion has been reported to involve direct transport via a pore (although it is unclear whether the pore forming protein is at the plasma membrane or elsewhere), by autophagy dependent pathway, by the involvement of GRASPs and ER specific IRE1 and PERK, and by pyroptosis. The field at present is riddled with issues and we have decided to focus only on the starvation specific secretion in yeast. Testing kinases reported for other pathways will not help unless we know the targets and therefore, we kindly beg the reviewers that we are excused from taking this undertaking.

We have also now included the following statement in the discussion to highlight the involvement of ROS, mitochondria and IL-1ß secretion. "ROS and mitochondrial function have been shown to control IL-1ß secretion in mammalian cells (Gabelloni et al., 2013; Jabaut et al., 2013; Zhou et al., 2011)".

3. Specify the abundance of the ROS-related hits, and also show specificity by examining secretion (or lack thereof) of other abundant cytosolic enzymes (Reviewer 1, point 2; Reviewer 2, point 1)

We now show that proteins secreted in a ROS dependent manner do not undergo any obvious change in their abundance upon starvation. Moreover, the entire proteome data that we have now shown (Figure S1) reveals that only 2 proteins show more than 2 fold increase and 9 show a more than 2 fold decrease in abundance. These proteins that show a change in their abundance are not secreted unconventionally and therefore linked to other cytoplasmic events triggered by starvation. We thank the reviewers to help us undertake this exercise. This has helped us in stating that cells do not undergo a drastic change in their overall proteome during starvation for the period of our experimental procedures.

We have calculated the percent of Ahp1, Trx1 and Trx2 secreted upon starvation. Like previously reported for SOD1 and Acb1, they are secreted in very low amounts (less than 1%). This is now mentioned in the main text and the figure legends have been modified to detail the relative loading amounts so this fact is more apparent.

Please note, the new SILAC mass spectrometry data has been uploaded to the ProteomeXchange Consortium, along with the secretome data, with the dataset identifiers PXD010849 (secretome) and PXD016815 (SILAC). This information is also included in the materials and methods section.
Reviewer access: Secretome: ***Username:*** *reviewer12862@ebi.ac.uk,* ***Password:*** *NC2NFQY0*
SILAC: ***Username:*** *reviewer63707@ebi.ac.uk,* ***Password:*** *WEPXCDqI*

4. Provide some additional characterization of mitochondrial function/behaviour as suggested by Reviewers 2 and 3. Please use your discretion in determining the specific experiments that would best address these concerns.

We appreciate the reviewers' insights and advice, but this paper is not so much about mitochondrial form and function, but related to production of enzymatic levels of ROS upon starvation. Also, in our new version, we have arranged the text to highlight the significance of ROS generation without going into the details of changes in mitochondrial physiology during starvation. We have pointed out that the change to large, round morphology is reminiscent of that seen in ERMES mutants, and we suggest mitochondrial function may be perturbed, but this is beyond the scope of this paper.

In sum, we present a simple proposal that ROS produced during starvation triggers the release of many signal sequence lacking proteins that predominantly compose antioxidants in addition to known signaling proteins like Acb1. These proteins require the activity of Grh1, which further attests to the central role of Grh1 in unconventional protein secretion. The loss of Grh1 causes a defect in cell growth as evident by the number of colonies that grow from starving cells lacking Grh1 compared to wild type cells. These data will be of interest to scientists interested in ROS, Grh1, unconventional protein secretion, Lipogenic activities, antioxidants like SOD1, and in general protein secretion.

Thanks for your advice and assistance,

Vivek Malhotra and Amy Curwin.

January 8, 2020

RE: JCB Manuscript #201905028R

Dr. Amy J Curwin
Centre for Genomic Regulation

Dear Dr. Curwin:

Thank you for submitting your revised manuscript entitled "Reactive oxygen species (ROS) triggers unconventional secretion of antioxidants and Acb1". The original reviewers #1 and #3 have now assessed the paper and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

Please be sure to address the final (minor) comments of these two reviewers and please be sure to provide a point-by-point rebuttal along with your final revision.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis - this includes cropped gels like those in figures 2D, 2E,and 3C.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

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,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

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

The revised manuscript is improved. The data are high quality and of interest, but the novelty remains somewhat modest as several other papers (now cited as requested near the end) have reported a connection between IL-1beta secretion and ROS and mitochondrial function. The novelty relates to identification of a set of proteins whose release is vps23 dependent and coordinated in response to ROS.

The authors should identify the proteins off the centerline on the volcano plot (Fig. S1) to aid the reader.
Page 10 line 11 refers to Fig. 3, not Fig. 2

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

With revisions to their original manuscript, Cruz-Garcia and colleagues have addressed several important concerns that were raised during the initial review.

1. Regarding physiological significance of unconventional secretion of enzymes with anti-oxidant activities, the authors now show that mutations that diminish unconventional secretion result in reduced viability of the cells after starvation. These new data do show that NAC substantially protects grd1 and vps23 deletion cells from starvation-induced loss of viability.

2. Agreed - the unconventional secretion literature is complicated to cite in an understandable manner (for the unfamiliar reader). I am fine with the modest revisions.

3. The SILAC experiment shows that the abundances of the unconventionally secreted proteins do not change significantly during starvation.
Hence, the increase in extracellular levels of these proteins is not due to increased abundance in the cell.

4. It's a bit disappointing that the consequences of starvation on mitochondria were not followed up. I agree to some extent that the slides outside the scope of this paper.

Minor points

In Figure 4A the ordinate axis labels are difficult to follow. Why indicate the exponential for every value? To me, it would be clearer to indicate fold differences.

Figure 4B: Please define "negative" in the figure legend.