THE EMBO JOURNAL

Host cell egress of Brucella abortus requires BNIP3L-mediated mitophagy

Jérémy Verbeke, Youri Fayt, Lisa Martin, Oya Yilmaz, Jaroslaw Sedzicki, Angéline Reboul, Michel Jadot, Patricia Renard, Christoph Dehio, Henri-François Renard, Jean-Jacques Letesson, Xavier De Bolle, and Thierry Arnould
DOI: 10.15252/embj.2022112817

Corresponding author(s): Thierry Arnould (Thierry.arnould@unamur.be)

Review Timeline:

Submission Date:	12th Oct 22
Editorial Decision:	7th Nov 22
Revision Received:	12th Apr 23
Editorial Decision:	5th May 23
Revision Received:	9th May 23
Accepted:	11th May 23

Editor: Ieva Gailite

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **7th Nov 2022**

Dear Dr. Arnould,

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, all reviewers find the presented role of mitophagy in regulation of Brucella abortus egress and formation of Brucella-containing mitochondrial compartments (mBCVs) very interesting. However, they also raise a number of issues that would need to be addressed in a revision, in particular regarding conservation of mitophagy induction and mBCV formation in macrophages (referees #1 and #2), the contribution of mBCVs to B. abortus egress (referees #1 and #2), and further investigation of the BNIP3L role in B. abortus infection (referee #3, point 1). Based on these positive assessments, I would like to invite you to address the issues raised by the reviewers in a revised manuscript. I think it would be useful to discuss the revision in more detail via email or phone/videoconferencing - please let me know which option you prefer.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please contact us to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Ieva Gailite

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).

- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (5th Feb 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

This study shows that Brucella-induced mitochondrial fragmentation is associated with enhanced mitophagy, which induces the formation of the specialized compartments called aBCVs mediating bacterial egress. The authors show this process depends on the receptor BNIP3L, controlled by HIF1 alpha, not in response to hypoxia but to iron starvation induced during infection. Overall these findings are major discoveries for the Brucella-pathogenesis field as it implicates, for the first time, mitophagy in the infectious cycle. In addition, these findings are of broader interest highlighting receptor-mediated mitophagy in bacterial pathogenesis.

Specific major concerns

1. There is a significant emphasis throughout the manuscript on the existence of mitochondrial Brucella-containing vacuoles (mBCVs) as a new Brucella intracellular compartment. Yet, these structures represent only a small proportion of BCVs at 72h post-infection in HeLa cells and have no apparent function (for example, in egress or mitophagy control). In addition, they are not aBCVs which are the ones controlled by mitophagy. Therefore, the relevance of these vacuoles is unclear.

- Are these mBCVs present in a more relevant infection model (as primary macrophages) to ensure they are not an artefact of immortalized HeLa cells?
- Could it be a simple consequence of mitophagy in an overcrowded cell?
- Are mBCVs contributing to egress? (this could be assessed by checking the presence of LAMP1 and mitochondrial markers on re-infection events).
- Is mBCV formation impacted by blocking Brucella-induced mitophagy (siBNIP3L)?

2. It is unclear how the authors see this process unfolding. Brucella-induced mitophagy contributes to the regulation of bacterial egress and aBCV formation, but at the same time, these aBCVs have no mitophagy/mitochondrial markers. If mitophagy directly controls aBCV formation, wouldn't one expect to detect mitochondrial labelling on aBCVs? This would certainly be the case if mitochondria were to provide membranes, as proposed in the discussion, which renders this hypothesis unlikely.

3. Could the effect of mitophagy on bacterial egress be an indirect consequence? In the discussion, the authors propose that mitophagy could provide membranes (see point 2) or contribute to Brucella inhibition of cell death. Did authors observe an enhanced loss of cells or the presence of apoptotic nuclei when blocking Brucella-induced mitophagy? Authors should test whether blocking the mitophagy (e.g. siBNPIP3L) changes the viability of the infected cells. If cells undergo cell death, the bacterial egress process may be indirectly halted.

Minor corrections

"We found that the number of LAMP-1-positive BCVs is significantly reduced in cells depleted for BNIP3L to a similar extent" (instead of extend)

Referee #2:

The intracellular bacterial pathogen Brucella abortus completes its infectious cycle by subverting host cell autophagic functions to form specific organelles (aBCVs) that are associated with bacterial egress. The mechanisms of aBCV formation and bacterial egress remain however unclear. Expanding upon their previous observations that Brucella infection of macrophages and epithelial cells causes mitochondrial fragmentation, Verbeke and colleagues report here that this process is associated with increased mitophagy at the aBCV stage and with formation of swollen, bacteria-containing mitochondria that they name mitochondrial BCVs (mBCVs). Furthermore, they show that Brucella-induced mitophagy is Parkin-independent but dependent upon the mitophagy receptor BNIP3L, which is induced by iron starvation-dependent stabilization of the hypoxia-regulated factor HIF-1a and is also required for aBCV formation and bacterial egress.

This is a well performed study that reports intriguing new observations of mitochondrial interactions with Brucella and provides compelling evidence of BNIP3L-mediated mitophagy induction at a late stage of the Brucella infectious cycle, a process that could be triggered via iron depletion and that contributes to aBCV formation and bacterial egress. This work has therefore a strong potential to provide significant advances in our understanding of the mechanisms of Brucella egress from infected cells but also of how pathogens manipulate mitochondrial functions and homeostasis. Some aspects of this study deserve some clarifications, however, as the functional connections between BNIP3L-dependent mitophagy, mBCVs and bacterial egress remain confusing, which limits the overall impact of the study. In particular, this manuscript would strongly benefit from establishing whether mBCVs are formed via iron starvation-induced, BNIP3L-dependent mitophagy and what their contribution to bacterial egress is.

Major comments.

1. The authors convincingly demonstrate that Brucella infection causes mitochondrial fragmentation and BNIP3L-dependent mitophagy and that BNIP3L is required for aBCV formation and bacterial egress, yet they do not establish the role of mBCVs in bacterial egress, especially considering that they appear distinct from LAMP1+ aBCVs (Figure 7F). I think it is important to the coherence and impact of the study that the authors test whether BNIP3L depletion inhibits mBCV formation, as would be expected if they contribute to bacterial egress.

2. The data showing that iron supplementation prevents activation of the HIF-1a/BNIP3L pathway is impressive but falls short of testing whether iron starvation is the actual trigger of aBCV formation and bacterial egress. Could the authors test whether iron supplementation inhibits aBCV formation, mBCV formation and bacterial egress? Establishing such a connection would also reinforce the authors' argument that this mitophagy pathway controls bacterial egress.

3. HeLa cells are classically used in many studies of Brucella-host cell interactions, although they do not represent host cells that are primarily targeted during infection, i.e. myeloid cells such as macrophages. Since the authors previously established that mitochondrial fragmentation also occurs in macrophages-like RAW264.7 cells, I think it is important to the significance of the study that they test whether mitophagy and mBCVs form in macrophages and contribute to bacterial egress. Providing evidence of mBCV formation in macrophages and the testing the effect of BNIP3L depletion on aBCV formation/bacterial egress would likely address this concern.

Minor comments.

1. The role of BNIP3L in Brucella-induced mitophagy is interesting but is it the only of the several ubiquitin-independent mitophagy receptors involved? Have the authors tested depletion of others to evaluate BNIP3L specificity?

2. While aBCV formation does not require LC3, BNIP3L-induced mitophagy should include an LC3 recruitment step. In keeping with the idea that mBCV may result from these mitophagy events, have the authors tested whether mBCVs accumulate LC3?

3. The data excluding a Parkin-dependent process in Brucella-induced mitophagy is a little "light" for this journal, as it is only based on negative recruitment of Parkin to mitochondria in infected cells. Have the authors tested whether Parkin depletion affects Brucella-induced mitophagy?

4. Could mBCVs phenotypically become aBCVs through mitophagosomal maturation? Do degraded mitochondrial fragments through this pathway become TOMM20-negative and LAMP1-positive?

5. Fig. 4C-D. It is hard to see recruitment of BNIP3L to mitochondria in the figure presented. Perhaps adding a magnified inset of a representative area would be more informative?

6. Fig. 6A-C. Based on the role of BNIP3L in mitophagy, it is surprising that its depletion affects mitochondrial fragmentation and not mitophagy only. Could the authors discuss this point?

7. Fig. 7B and E. The suppressive effect of BNIP3L knockdown on Brucella reinfection is dramatic, but less so on aBCV

formation. Should proportionality not be expected in this case, or would the distinct population of mBCVs also contribute to bacterial egress? This could be partially addressed by establishing whether mBCV formation is BNIP3L-dependent (see major comments).

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

Verbeke and colleagues present compelling evidence that Brucella infection modifies mitochondrial network integrity, affecting BNIP3L-mediated mitophagy to impact pathogen egress. In general, the study is well conducted and presented. The Parkin-independent nature of the phenotype is convincing, and the identification of mitochondrial BCVs is exciting. In general, I am supportive of this manuscript and recommend, for improved clarity, that the findings presented should be discussed further with respect to pre-existing literature.

Specific major concerns essential to be addressed to support the conclusions

1. It would be beneficial to consolidate the effects of Brucella on mitophagy empirically. Pre-existing evidence exists that Brucella infection modifies the autophagy pathway (PMID: 9826346). Moreover, BNIP3L/NIX also act as 'eat-me' signals for peroxisomes to mediate pexophagy (which is also triggered by iron depletion - PMID: 36215693). To address this, the authors might want to consider the following:

a. Infection experiments with macroautophagy vs mitophagy reporters would uncouple these effects (e.g. mCherry-GFP-LC3). This will unambiguously clarify if the mitophagy events observed are attributable to macroautophagy.

b. Comparative profiling of peroxisomal vs mitochondrial mass (e.g. PMP70/Catalase (peroxisomes) and HSP60 (mitochondrial matrix) immunoblots). However, the reviewer appreciates that bulk protein analysis may not be appropriate if all cells are not infected upon B. aborteus treatment. Alternatively, an assessment of peroxisomal network architecture (PMP70 or catalase immunostaining) would prove informative.

2. HIF1-alpha stabilisation occurs rapidly in response to iron depletion (PMID: 32420530), and mitochondrial iron depletion exerts a range of highly specific metabolic changes leading to mitophagy (PMID: 35411952). The authors posit that Brucella infection triggers cellular iron depletion. Because mitochondria are a significant source of intracellular iron (PMID: 30485761), could Brucella infection deplete mitochondrial iron to launch NIX/BNIP3L and mitophagy? This would be impossible to test but should at least be considered by the authors as a possible mechanism in the revised manuscript. The intimate association between Brucella and the iron-rich mitochondrial network would make complete sense and refine assumptions about the precise pool of labile iron affected by the pathogen.

3. The effects on the mitochondrial network are apparent, but can the authors comment if this phenotype reflects increased mitochondrial fission? Additionally, the authors present a very nice EM image in the paper. Does infection broadly affect the architecture, or are there discernible alterations to mitochondrial ultrastructure? If already performed in their previous article - it would help the reader for the present findings to be placed in the broader context. Can the authors comment in more explicit detail for the reader - is the mitochondrial fragmentation phenotype unique to Brucella infection, or is it something that happens during all infection paradigms? Some evolutionary perspectives would also bolster the revised discussion of this phenomenon.

Minor concerns that should be addressed

1. It would be helpful to understand the magnitude of the mitophagy response induced by Brucella relative to the canonical iron-depletion NIX/BNIP3-mediated response. An experiment with deferiprone (DFP) included as a positive control would inform the reader as to the extent of mitochondrial turnover in these cells.

2. A more comprehensive and precise discussion regarding the presence of B. abortus inside TOMM20 membranes should be included in the revised manuscript. Evidence suggests that mitochondria can shed TOMM20-positive vesicles (MDVs, that can be signalling or degradative) and even contribute membrane to autophagosomes in distinct contexts, and PINK1/Parkin-play a role in MDV formation. Because the authors utilised HeLa cells which are devoid of endogenous Parkin, this is likely not the mechanism at play. Nonetheless, how do they get there? In their discussion - can the authors signpost important experimental avenues to dissect this in future studies?

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

- In the introduction, it is stated that canonical, ubiquitin-dependent mitophagy depends on the PINK1-Parkin pathway. This assumption is valid only for cell lines and is not supported by the in vivo evidence. The introduction should be updated to reflect the current state of the art, and a distinction should be made between the basal and stress-induced pathways. This would also

flow nicely with the Parkin-independent phenotypes reported in their paper.

- "During mitophagy, targeted mitochondria interact with the autophagy protein LC3 inserted in autophagic phagophores, which are recruited around their cargo" - I suggest the use of 'ATG8-proteins', a term that also captures the GABARAP proteins which can participate in this process.

- Several mitophagy reporters exist-Allen et al. utilised mCherry-GFP-FIS1mt101-152 (commonly known as mito-QC). The authors refer to "FIS1-mCherry-GFP". Was mito-QC used, or is this a new FIS1-based reporter? Consistency would be helpful for the naming of the precise reporter used.

- Labelling in Figures 4-6 is unconventional (GFP is red, and HIF1-alpha is green etc.). This is not a dealbreaker, but as pixels are pseudo-coloured in any case, it may be helpful for readers if the colours were reassigned to their corresponding conventions in the representative images within the figures.

- A more streamlined figure 8 (step-by-step) may improve clarity for the uninitiated reader (at present, it is aesthetic but takes a while to figure out).

- Violin plots combined with the scatterpoints may be more informative for statistical interpretation of data distribution.

Referee #1:

This study shows that Brucella-induced mitochondrial fragmentation is associated with enhanced mitophagy, which induces the formation of the specialized compartments called aBCVs mediating bacterial egress. The authors show this process depends on the receptor BNIP3L, controlled by HIF1 alpha, not in response to hypoxia but to iron starvation induced during infection. Overall these findings are major discoveries for the Brucella-pathogenesis field as it implicates, for the first time, mitophagy in the infectious cycle. In addition, these findings are of broader interest highlighting receptor-mediated mitophagy in bacterial pathogenesis.

- We first would like to thank the reviewer #1 for his/her positive assessment of our study and manuscript as well as useful comments and suggestions that helped us to improve our manuscript. As detailed below, we have addressed all the concerns raised and present now a revised version of the work.

Specific major concerns

1. There is a significant emphasis throughout the manuscript on the existence of mitochondrial *Brucella*-containing vacuoles (mBCVs) as a new *Brucella* intracellular compartment. Yet, these structures represent only a small proportion of BCVs at 72h post-infection in HeLa cells and have no apparent function (for example, in egress or mitophagy control). In addition, they are not aBCVs which are the ones controlled by mitophagy. Therefore, the relevance of these vacuoles is unclear.

- We totally agree with this comment that the role of the mBCVs still needs to be elucidated since we never claimed to find the function of these newly discovered structures yet. It would be indeed very interesting to investigate their putative role in the last steps of *Brucella* intracellular cycle. Further research in line with this comment was therefore performed.

- Are these mBCVs present in a more relevant infection model (as primary macrophages) to ensure they are not an artefact of immortalized HeLa cells?

- Indeed, despite being really convenient for organelle morphology analyses, HeLa cells are far from being the most relevant model in the context of *Brucella* infection. Since macrophages represent a more relevant *in vitro* model, immortalised bone marrow-derived macrophages (iBMDMs) were infected by the bacteria to analyse and quantify the occurrence of mBCVs. As shown in Fig. R1 below, mBCVs are also observed in iBMDMs at 48 h pi and more frequently at 72h pi. This result reinforces the relevance of this new type BCVs in other cell type than HeLa cells. These results are now included in the revised version of the manuscript and are presented in Fig. 8A-C.

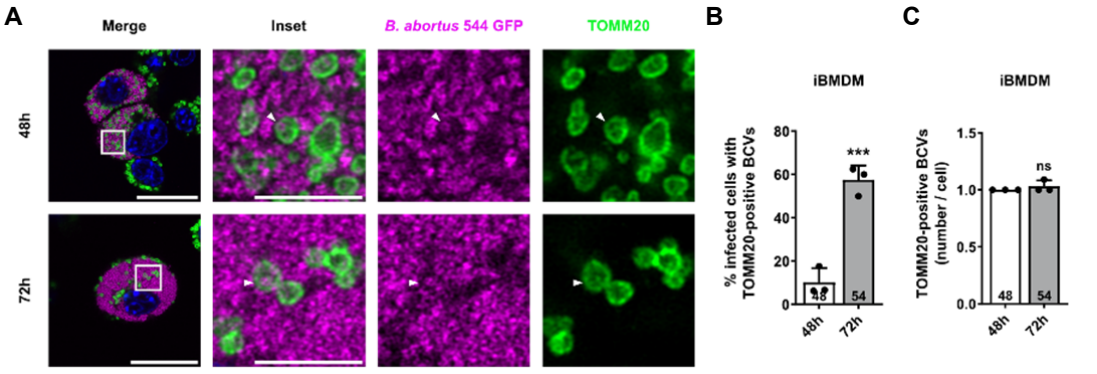

Figure R1. *B. abortus* is observed in mitochondria of iBMDM.

A. Representative confocal micrographs of iBMDM infected with *B. abortus* 544 GFP (Magenta) for 48 and 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 647 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset scale bars: 5 µm.
B. Quantification of the percentage of infected iBMDM displaying TOMM20-positive BCVs (mBCVs) at the indicated times, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates)

independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ***: p <0.001 (p = 0.0009).
C. Quantification of the number of TOMM20-positive BCVs (mBCVs) per infected HeLa cells, at the indicated times, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ns: not significant (p = 0.3739).

- Could it be a simple consequence of mitophagy in an overcrowded cell?

- The reviewer suggested thus that mBCVs could come from the mitophagy process which takes place upon *Brucella* infection. This is potentially an interesting hypothesis. Indeed, during mitochondrial membrane degradation, the bacteria could enter the mitochondrial structure through a breach in their membrane and could thus enter inside the mitochondria. However, this would means that (1) the integrity of mitochondria ultrastructure would be highly altered and (2) mBCVs would need to be surrounded by autophagic membranes. None of these features could be observed in the FIB/SEM experiments in Fig.8G of the revised manuscript.

- Nonetheless, and as Referee #3 has also suggested that possibility (see below), the putative co-localisation between LC3 and the mBCVs was tested to determine whether mBCVs could result from *Brucella*-induced mitophagy in a process of mitophagosome formation, or not. As shown in Fig. R2 below, no co-localisation between LC3 and the TOMM20-positive BCVs (mBCVs) could be observed. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5F

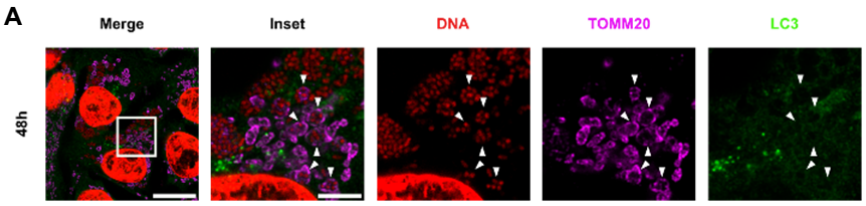

Figure R2. LC3 does not seem to colocalise with TOMM20 positive mBCVs.

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 WT for 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 633 – Magenta) and LC3 (Alexa Fluor 568 – Green). DNA (from the HeLa nucleus and *B. abortus*) was stained with Hoechst 33258 (Red Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset zoom scale bars: 5 µm.

- Is mBCV formation impacted by blocking *Brucella*-induced mitophagy (siBNIP3L)?

- To address this question, we tested whether BNIP3L depletion (using siBNIP3L) could affect mBCV formation. As shown in Fig. R3 below, the depletion of BNIP3L does not affect the number of mBCVs counted in HeLa cells at 72 h pi. These results strongly suggest that mBCVs are not related to the mitophagy response triggered by *B. abortus*. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5B-C.

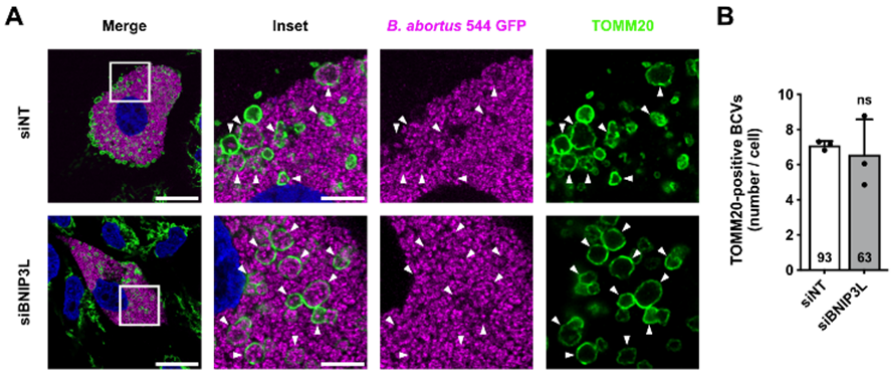

Figure R3. BNIP3L depletion does not alter mBCV occurrence.

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 GFP (Magenta) and transfected with a non-targeting siRNA pool (siNT – 40 nM) or a BNIP3L siRNA SMARTpool (siBNIP3L – 40 nM) for 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 647 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset scale bars: 5 µm.
B. Quantification of the number of TOMM20-positive BCVs (mBCVs) per infected HeLa cells, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ns: not significant (p = 0.6742).

- Are mBCVs contributing to egress? (this could be assessed by checking the presence of LAMP1 and mitochondrial markers on re-infection events).

- We fully agree with the referee's suggestion that one putative function of mBCVs might be linked to bacterial egress and/or aBCV formation as mBCVs seem to be abundant at 72 h pi. However, this would means that *Brucella* would keep its BCV membrane during egress and re-infection of new cells, which is not described yet. Moreover, as we showed that mBCVs occurrence is not related to the *B. abortus*-induced BNIP3L-mediated mitophagy, it is unlikely that mBCVs are contributing to bacterial egress. Nevertheless, and as suggested by the reviewer, we performed LAMP1 and TOMM20 co-immunostainings on re-infected cell but we never observed neither mBCVs nor LAMP-1/TOMM20-related markers on early events of re-infection. Since the study of the mechanisms of membrane turnover during aBCV formation and bacterial egress has never been investigated, it was difficult for us to conclude whether the lack of markers was observed because of a technical issue, or because *Brucella* does not keep a membrane during egress, which would be true if the aBCV membrane fuses with the host plasma membrane during bacterial egress.

2. It is unclear how the authors see this process unfolding. *Brucella*-induced mitophagy contributes to the regulation of bacterial egress and aBCV formation, but at the same time, these aBCVs have no mitophagy/mitochondrial markers. If mitophagy directly controls aBCV formation, wouldn't one expect to detect mitochondrial labelling on aBCVs? This would certainly be the case if mitochondria were to provide membranes, as proposed in the discussion, which renders this hypothesis unlikely.

- As BNIP3L-mediated mitophagy controls *Brucella* egress and aBCV occurrence at 72 h pi for still unclear mechanistically reasons, we never stated for a direct link between these two processes. We agree that the aBCVs are negative for TOMM20 (the only mitochondrial marker tested in our study), but it does not exclude the presence of other OMM proteins, or in another view, the loss of some mitochondrial proteins during membrane turnover. A screening of other OMM proteins could be performed to better characterize the markers of aBCVs, which are to date quite limited, including only the well-described LAMP-1 protein. Moreover, the link between autophagy and aBCV formation seems indirect/incomplete regarding the fact that only some autophagy initiation proteins (ULK1, Beclin, ATG14L1), but not autophagy elongation proteins, are important for aBCV formation (Starr *et al*, 2008). Thus, the direct contribution of autophagy in aBCV formation still need to be elucidated. Similarly, the link between mitophagy and aBCV formation might be more complex than just a direct consequence. One could also hypothesize that the elimination of damaged mitochondria (and related damages) could indirectly help the formation of aBCV and fulfilment of *Brucella* infection cycle.

  A comment about this topic was added in the discussion section of the revised manuscript (lines 371-381).

3. Could the effect of mitophagy on bacterial egress be an indirect consequence? In the discussion, the authors propose that mitophagy could provide membranes (see point 2) or contribute to Brucella inhibition of cell death. Did authors observe an enhanced loss of cells or the presence of apoptotic nuclei when blocking *Brucella*-induced mitophagy? Authors should test whether blocking the mitophagy (e.g. siBNPIP3L) changes the viability of the infected cells. If cells undergo cell death, the bacterial egress process may be indirectly halted.

- Indeed, and as mentioned in the manuscript, mitophagy induced by *Brucella* could inhibit the cell death of the host for bacterial survival. In this case, we might suspect that apoptosis could be induced in absence of mitophagy (siBNIP3L). However, neither apoptotic nuclei nor a loss or decreased number of infected cells were ever observed upon BNIP3L depletion in infected cells, as you can observe on Fig. 5 and 6 (referring to the micrographs from siBNIP3L experiments) in the revised manuscript.

- As cell death could take several forms such as necrosis, necroptosis, pyroptosis, autophagy or ferroptosis, a more general test using a live/dead kit (based on changes in membrane permeability – Fixable viability dye eFluor506, eBiosciences, ThermoFisher) for flow cytometry analysis was also performed to directly test host cell viability upon BNIP3L depletion in infected cells. As shown in Fig. R4 below, the viability of infected cells treated with the siNT or the siBNIP3 is similar, and the related signal of the fixable viability dye is below the threshold of control cells for which membrane permeability has been compromised by boiling the cells for 5 min. This result suggests that BNIP3L depletion does not seem to alter the viability of infected cells, arguing against the fact that the effect mitophagy on bacterial egress could be an indirect consequence/effect.

A

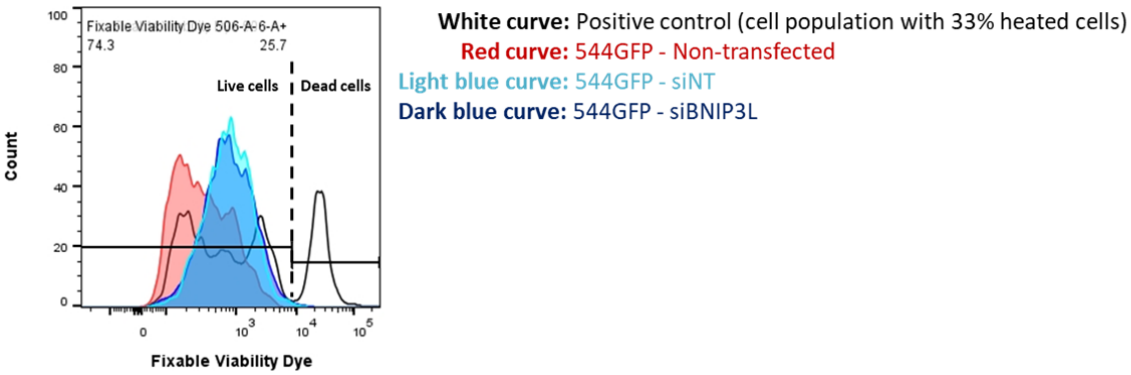

Figure R4. BNIP3L depletion does not alter the viability of infected cells.

A. Representative fluorescence intensities of the fixable viability Dye 506 from HeLa cells infected with *B. abortus* 544 GFP and transfected with a non-targeting siRNA pool (siNT – 40 nM) or a BNIP3L siRNA SMARTpool (siBNIP3L – 40 nM) for 48 h as measured by flow cytometry. A sample in which 33 % of the cell population was boiled for 5 min was used as a positive control (white curve) and determined the threshold above which cells can be considered as displaying disturbed membrane permeability and therefore cell death.

- In addition, previous experiments performed in our lab assessing for the presence of the active form of the caspase 3 revealed that mitochondrial fragmentation does not protect *Brucella*-infected macrophages from TNFα-induced apoptosis (Lobet *et al*, 2018). This observation is an additional argument to suggest that *Brucella*-induced mitophagy probably does not contribute to the inhibition of host cell death.

- Regarding these several arguments, the hypothesis of a putative regulation of the host cell death by *Brucella* through mitophagy was removed from the revised manuscript.

Minor corrections

"We found that the number of LAMP-1-positive BCVs is significantly reduced in cells depleted for BNIP3L to a similar extent" (instead of extend)

- The misprint was corrected in the revised manuscript (line 242).

Referee #2:

The intracellular bacterial pathogen Brucella abortus completes its infectious cycle by subverting host cell autophagic functions to form specific organelles (aBCVs) that are associated with bacterial egress. The mechanisms of aBCV formation and bacterial egress remain however unclear. Expanding upon their previous observations that *Brucella* infection of macrophages and epithelial cells causes mitochondrial fragmentation, Verbeke and colleagues report here that this process is associated with increased mitophagy at the aBCV stage and with formation of swollen, bacteria-containing mitochondria that they name mitochondrial BCVs (mBCVs). Furthermore, they show that Brucella-induced mitophagy is Parkin-independent but dependent upon the mitophagy receptor BNIP3L, which is induced by iron starvation-dependent stabilization of the hypoxia-regulated factor HIF-1a and is also required for aBCV formation and bacterial egress.

This is a well performed study that reports intriguing new observations of mitochondrial interactions with *Brucella* and provides compelling evidence of BNIP3L-mediated mitophagy induction at a late stage of the *Brucella* infectious cycle, a process that could be triggered via iron depletion and that contributes to aBCV formation and bacterial egress. This work has therefore a strong potential to provide significant advances in our understanding of the mechanisms of *Brucella* egress from infected cells but also of how pathogens manipulate mitochondrial functions and homeostasis. Some aspects of this study deserve some clarifications, however, as the functional connections between BNIP3L-dependent mitophagy, mBCVs and bacterial egress remain confusing, which limits the overall impact of the study. In particular, this manuscript would strongly benefit from establishing whether mBCVs are formed via iron starvation-induced, BNIP3L-dependent mitophagy and what their contribution to bacterial egress is.

- We thank the reviewer #2 for his/her enthusiastic evaluation and agree with the concern about the question of a putative link between BNIP3L-mediated mitophagy, mBCVs and bacterial egress. As developed below, we now provide new experimental evidence of a putative role of mBCVs.

Major comments.

1. The authors convincingly demonstrate that *Brucella* infection causes mitochondrial fragmentation and BNIP3L-dependent mitophagy and that BNIP3L is required for aBCV formation and bacterial egress, yet they do not establish the role of mBCVs in bacterial egress, especially considering that they appear distinct from LAMP1+ aBCVs (Figure 7F of the previous version). I think it is important to the coherence and impact of the study that the authors test whether BNIP3L depletion inhibits mBCV formation, as would be expected if they contribute to bacterial egress.

- We fully agree with referee #2 who has the same comment and demand as the referee #1 on further exploration of the effect of BNIP3L on the formation of mBCVs. As suggested by referees #1 and #2, we thus tested whether BNIP3L depletion (using siBNIP3L) decreased the occurrence of mBCVs or not. As shown in Fig. R3 below (already showed in response to referee #1), we showed that BNIP3L depletion does not affect the number of mBCVs in HeLa cells at 72 h pi. These results strongly suggest that mBCVs are not related to the mitophagy response triggered by *B. abortus*. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5B-C.

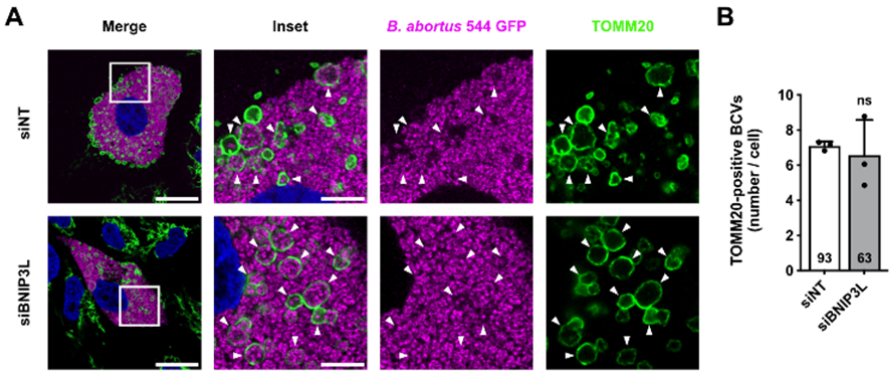

Figure R3. BNIP3L depletion does not alter mBCV occurrence. (Reproduced from referee #1's section)

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 GFP (Magenta) and transfected with a non-targeting siRNA pool (siNT – 40 nM) or a BNIP3L siRNA SMARTpool (siBNIP3L – 40 nM) for 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 647 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset scale bars: 5 µm.
B. Quantification of the number of TOMM20-positive BCVs (mBCVs) per infected HeLa cells, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ns: not significant (p = 0.6742).

2. The data showing that iron supplementation prevents activation of the HIF-1a/BNIP3L pathway is impressive but falls short of testing whether iron starvation is the actual trigger of aBCV formation and bacterial egress. Could the authors test whether iron supplementation inhibits aBCV formation, mBCV formation and bacterial egress? Establishing such a connection would also reinforce the authors' argument that this mitophagy pathway controls bacterial egress.

- We agree with the referee about the importance of showing whether bacterial egress is also iron-dependent and linked to the HIF-1 alpha/BNIP3L-mitophagy axis. As suggested, we tested whether $FeCl_2$ supplementation could also inhibit the occurrence of re-infection foci and aBCVs or not. As shown in Fig. R5 below, $FeCl_2$ supplementation also induces a drastic decrease in the percentages of reinfection foci, as well as a decrease in the occurrence of aBCVs at 72h pi. These results suggest that iron supplementation mimics the effects obtained with the siRNA-mediated depletion of BNIP3L, which indeed reinforces the argument that the iron/HIF-1alpha/BNIP3L-mediated mitophagy controls bacterial egress. These results are now included in the in the revised version of the manuscript and presented in Fig. 7A-D.

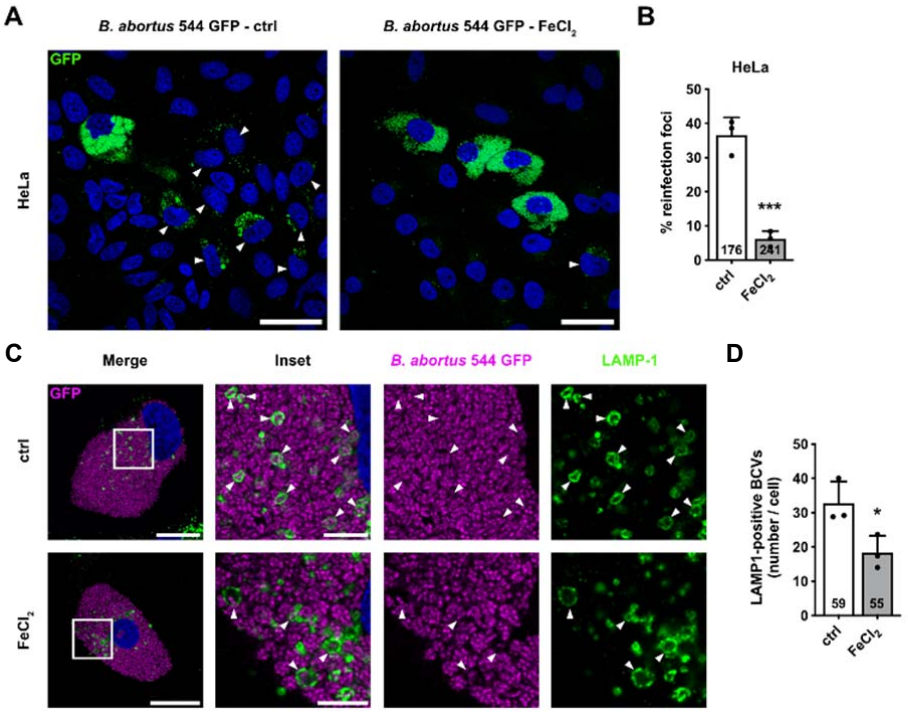

Figure R5. Iron limits aBCV formation and prevents reinfection events in HeLa cells.

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 GFP (Green) treated or not (ctrl) with $FeCl_2$ (500 µM) for 48 h, then incubated under reinfection-permissive conditions (with or without $FeCl_2$) for 24 h before analysis at 72 h pi. Cells were fixed and DNA was stained with Hoechst 33258 (Blue). Arrows indicate reinfected cells. Scale bars: 50 µm.

B. Quantification of the percentages of reinfection foci per infected cell at 72 h pi of HeLa cells from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ***: p <0.001 (p = 0.0008).
C. Representative confocal micrographs of HeLa cells infected or not with *B. abortus* 544 GFP (Magenta) treated or not (ctrl) with $FeCl_2$ (500 µM) for 72 h, then fixed and immunostained for LAMP-1 (Alexa Fluor 568 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate LAMP-1-positive BCVs (aBCVs). Scale bars: 20 µm. Inset zoom scale bars: 5 µm.
D. Quantification of the number of LAMP-1-positive BCVs (aBCVs) per infected HeLa cells from micrographs shown in (C). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; *: p <0.05 (p = 0.0368).

- In addition, we also tested whether iron supplementation could decrease (or alter) mBCV formation. In contrary to the effects observed for bacterial egress and aBCV formation, but in agreement with previous results we obtained showing that BNIP3L depletion does not alter mBCV occurrence, our results (Fig. R6 below) show that $FeCl_2$ supplementation does not significantly alter the number of mBCVs in infected cells at 72 h pi. These results reinforce the fact that mBCVs are most probably/likely not related to the BNIP3L-dependent mitophagy induced by *Brucella*. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5D-E.

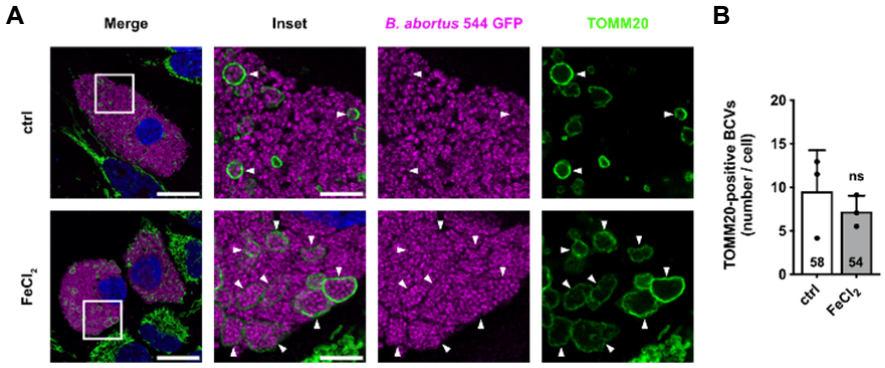

Figure R6. Iron supplementation does not alter mBCV occurrence in HeLa cells.

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 GFP (Magenta), treated or not (ctrl) with $FeCl_2$ (500 µM) for 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 647 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset zoom scale bars: 5 µm.
B. Quantification of the number of TOMM20-positive BCVs (mBCVs) per infected HeLa cells, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ns: not significant (p = 0.4724).

3. HeLa cells are classically used in many studies of *Brucella*-host cell interactions, although they do not represent host cells that are primarily targeted during infection, i.e. myeloid cells such as macrophages. Since the authors previously established that mitochondrial fragmentation also occurs in macrophages-like RAW264.7 cells, I think it is important to the significance of the study that they test whether mitophagy and mBCVs form in macrophages and contribute to bacterial egress. Providing evidence of mBCV formation in macrophages and the testing the effect of BNIP3L depletion on aBCV formation/bacterial egress would likely address this concern.

- We agree with the reviewer's comment (also mentioned by referee #1) that the HeLa cell line is not the most relevant model for studying *Brucella* intracellular trafficking. New experimental approaches were performed to confirm our results and observations in immortalized bone marrow-derived macrophages (iBMDMs). As suggested by referee #1 and #2, we tested whether mBCVs could also be observed in infected macrophages or not, and whether BNIP3L could be involved in the formation of mBCVs.

➢ First, as shown in Fig. R1 below (already showed in response to referee #1), we confirmed that mBCVs are also observed in iBMDMs at 48 and 72 h pi. These results are now included in the in the revised version of the manuscript and presented in Fig. 8A-C.

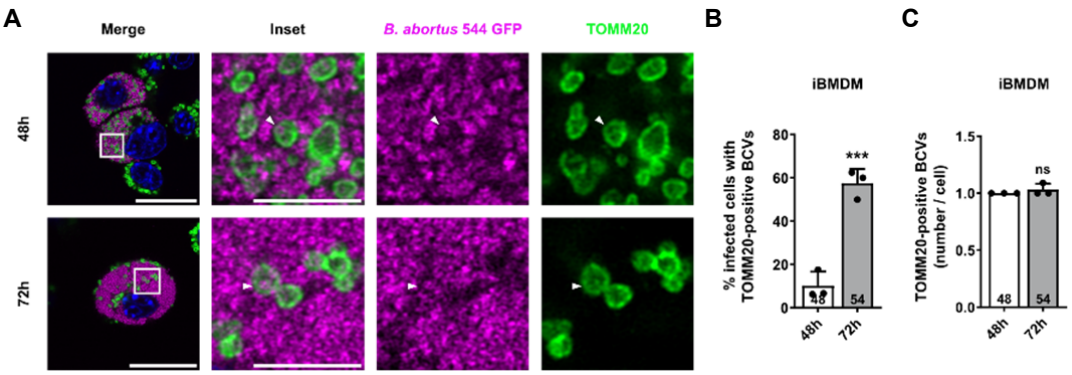

Figure R1. *B. abortus* is observed in mitochondria of iBMDM. (Reproduced from referee #1's section)

A. Representative confocal micrographs of iBMDM infected with *B. abortus* 544 GFP (Magenta) for 48 and 72 h, then fixed and immunostained for TOMM20 (Alexa Fluor 647 – Green). DNA was stained with Hoechst 33258 (Blue). Arrows indicate when *B. abortus* is found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset zoom scale bars: 5 µm.
B. Quantification of the percentages of infected iBMDM displaying TOMM20-positive BCVs (mBCVs) at the indicated times, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ***: p <0.001 (p = 0.0009).
C. Quantification of the numbers of TOMM20-positive BCVs (mBCVs) per infected HeLa cells, at the indicated times, from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; ns: not significant (p = 0.3739).

➢ To assess the effect of BNIP3L depletion on bacterial egress in macrophages, we first needed to validate the efficiency of the siRNA-mediated BNIP3L silencing in iBMDMs. Unfortunately, even if we tried several transfection reagents, we encountered many troubles/difficulties in obtaining an efficient BNIP3L downregulation, maintained overtime (see WB, Fig. R7 below). As an absence of a phenotype in response to a non-efficient gene silencing mediated by siRNA cannot be interpreted (as it could be either because the gene product does not contribute to the phenotype of interest or due to the fact that residual amount of the protein is still enough to contribute), we needed to reconsider our experimental strategy to answer this important question.

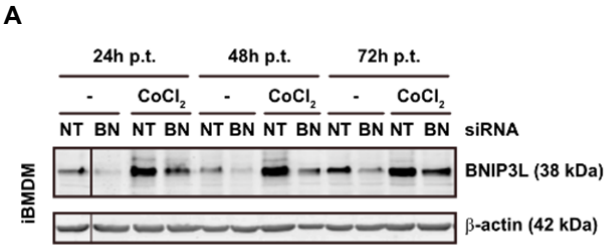

Figure R7. BNIP3L depletion efficiency in iBMDM using a siRNA SMARTpool approach.

A. Western-blot analysis of BNIP3L abundance in iBMDM transfected with a non-targeting siRNA pool (siNT – 40 nM) or a BNIP3L siRNA SMARTpool (siBNIP3L – 40 nM) for 24 h, then left for the indicated times post-transfection (p.t.) and treated (+) or not (-) with 100 µM $CoCl_2$ for 16 h before analysis. The abundance of β-actin was used as a loading control. Black bars indicate a crop in the membrane.

➢ In fact, as iron supplementation could mimic the effects obtained with an efficient siRNA-mediated BNIP3L silencing in HeLa cells, we hypothesised that comparable effect could be obtained for infected iBMDMs. Even if less specific, this approach could thus bypass the problems we encountered regarding the poor efficiency of a siRNA-mediated approach in iBMDMs, a general problem encountered in the scientific community as we discovered by discussing with several researchers in the field. After we confirmed that *B. abortus* also induces an iron-dependent expression of BNIP3L in iBMDMs (Fig. R8 below), we tested whether iron supplementation could prevent bacterial egress as observed in HeLa cells. As shown in Fig. R9 below, we showed that $FeCl_2$ supplementation decreases the percentages of reinfection foci in infected iBMDMs, reinforcing the significance of the observations in a more relevant *in vitro* model for *Brucella* infection and trafficking. These results are now included in the in the revised version of the manuscript and presented in Fig. 7E-F.

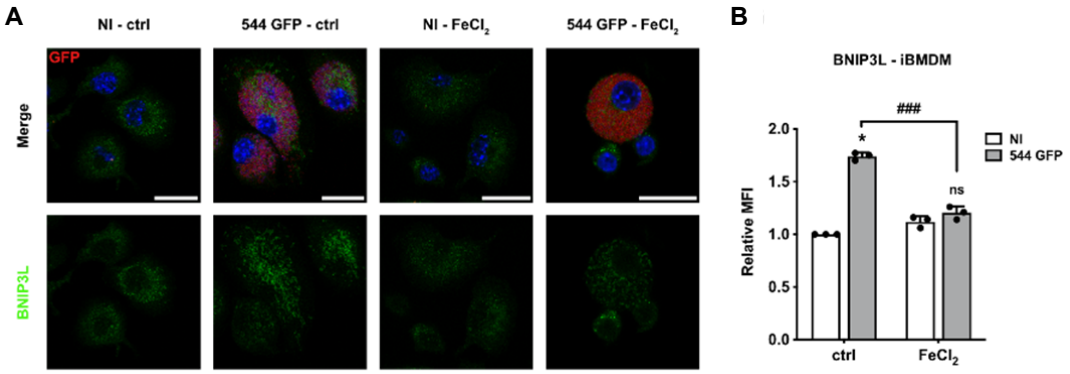

Figure R8. Iron prevents *B. abortus* induction of BNIP3L expression in iBMDM.

A. Representative confocal micrographs of iBMDM infected or not (NI) with *B. abortus* 544 GFP (Red) treated or not (ctrl) with 500 µM $FeCl_2$ for 48 h, then fixed and immunostained for BNIP3L (Alexa 568 – Green). DNA was stained with Hoechst 33258 (Blue). Scale bars: 20 µm.
B. Relative median fluorescence intensities (MFI) of BNIP3L immunostaining from iBMDM infected or not (NI) with *B. abortus* 544 GFP treated or not (ctrl) with 500 µM $FeCl_2$ for 48 h as measured by flow cytometry. Data are presented as means ± SD from n=3 (biological replicates) independent experiments (14,152 cells analysed in total per condition). Statistical analyses were performed using a two-way ANOVA followed by a Šidàk's multiple comparisons test; asterisks indicate significant differences compared to the control (NI); ns: not significant; *: p <0,05; hashtags indicate significant differences compared to the infected condition without $FeCl_2$; $^{###}$: p <0.001.

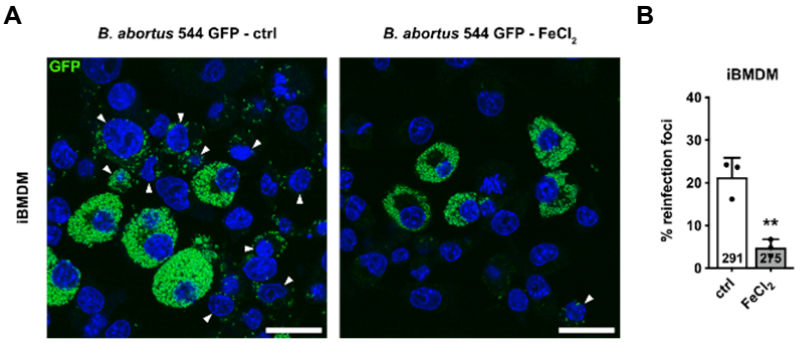

Figure R9. Iron prevents reinfection events in iBMDM.

A. Representative confocal micrographs of iBMDM infected with *B. abortus* 544 GFP (Green) treated or not (ctrl) with $FeCl_2$ (500 µM) for 48 h, then incubated under reinfection-permissive conditions (with or without $FeCl_2$) for 24 h before analysis at 72 h pi. Cells were fixed and DNA was stained with Hoechst 33258 (Blue). Arrows indicate reinfected cells. Scale bars: 50 µm.
B. Quantification of the percentages of reinfection foci per infected cell at 72 h pi of iBMDM from micrographs shown in (A). Data are presented as means ± SD from n=3 (biological replicates) independent experiments (the numbers indicated in the columns represent the number of cells analysed per condition). Statistical analyses were performed using an unpaired two-tailed Student's t-test; **: p <0.01 (p = 0.0043).

Minor comments.

1. The role of BNIP3L in *Brucella*-induced mitophagy is interesting but is it the only of the several ubiquitin-independent mitophagy receptors involved? Have the authors tested depletion of others to evaluate BNIP3L specificity?

- We thank the reviewer for that comment. Indeed, our results and observations do not exclude the putative role of other mitophagy receptors such as FUNDC1 which is also known to be induced by HIF1 upon HIF-1 alpha stabilisation. The depletion of other mitophagy receptors was eventually not tested. However, as the effect of BNIP3L depletion is almost complete on mitophagy readouts (the number of FIS1-mCherry-positive fragments is comparable to those found in cells maintained in control conditions), and very severe on mitochondrial morphology (the network is more connected and elongated as observed in control conditions), we conclude/hypothesise that BNIP3L is most likely one of the main mitophagy actors involved in these phenotypes induced by *Brucella*.

  A comment about the putative importance of FUNDC1 in *B. abortus*-mediated mitophagy was added in the revised version of the manuscript in the discussion (lines 307-313).

2. While aBCV formation does not require LC3, BNIP3L-induced mitophagy should include an LC3 recruitment step. In keeping with the idea that mBCV may result from these mitophagy events, have the authors tested whether mBCVs accumulate LC3?

- Understanding whether mBCVs might result from *Brucella*-induced mitophagy or not was also suggested by referee #1. To answer this question, we studied the co-localisation of LC3 with mBCVs. As shown in Fig. R2 below (already showed in response to referee #1), no co-localisation between LC3 and the TOMM20-positive mBCVs was observed. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5F.

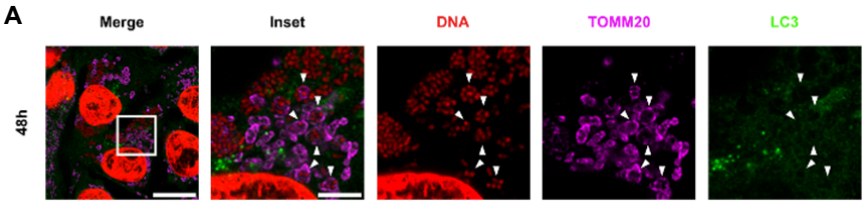

Figure R2. LC3 does not seem to colocalise with TOMM20 positive mBCVs. (Reproduced from referee #1' section)

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 WT for 72h, then fixed and immunostained for TOMM20 (Alexa Fluor 633 – Magenta) and LC3 (Alexa Fluor 568 – Green). DNA (from the HeLa nucleus and *B. abortus*) was stained with Hoechst 33258 (Red Arrows indicate when *B. abortus* was found inside a mitochondrion (mBCVs). Scale bars: 20 µm. Inset zoom scale bars: 5 µm.

3. The data excluding a Parkin-dependent process in *Brucella*-induced mitophagy is a little "light" for this journal, as it is only based on negative recruitment of Parkin to mitochondria in infected cells. Have the authors tested whether Parkin depletion affects *Brucella*-induced mitophagy?

- We thank the referee for this comment. However, since Parkin is not endogenously expressed in HeLa cells (Narenda *et al*., 2008, *J Cell Biol*), analysing Parkin depletion in this model turns out to be useless. Moreover, as we demonstrated, BNIP3L is one major actor in *Brucella*-induced mitophagy, a response observed in the absence of Parkin.

4. Could mBCVs phenotypically become aBCVs through mitophagosomal maturation? Do degraded mitochondrial fragments through this pathway become TOMM20-negative and LAMP1-positive?

- We thank the reviewer for this really interesting comment since there are physical connections between the ER (rBCV) and mitochondria. However, studying this putative transition might seem technically difficult. Indeed, this would require testing the putative co-localisation

between TOMM20 (mBCVs) and LAMP-1 (aBCVs) with mitophagy particles (FIS1-mCherry-GFP, already two colours) in addition with a *Brucella* marker (LPS staining since the GFP-expressing strain is not suitable with the FIS1 reporter). We would thus need 5 different colours, which we cannot perform with our microscopes. However, since we obtained new results during the reviewing process that clearly showed that mBCVs are not linked to BNIP3L mitophagy, we conclude that it is very unlikely that mBCVs could represent a transition/intermediate state between rBCVs and aBCVs.

5. Fig. 4C-D. It is hard to see recruitment of BNIP3L to mitochondria in the figure presented. Perhaps adding a magnified inset of a representative area would be more informative?

- A magnification of the BNIP3L-related signal was added in now Fig. 3C. In the revised version of the manuscript.

6. Fig. 6A-C. Based on the role of BNIP3L in mitophagy, it is surprising that its depletion affects mitochondrial fragmentation and not mitophagy only. Could the authors discuss this point?

- We fully agree with this referee's comment that it might be surprising to think that a mitophagy receptor is also directly responsible for mitochondrial fragmentation. Canonical mitophagy dynamics have intuitively led to the assumption that mitochondrial fission is a prerequisite for mitophagy because only small, fragmented mitochondria could be engulfed inside autophagosomes. However, recent reports indicate that mitophagy can still occur in cells having highly elongated networks (such as in DRP1 KO cells), and that mitochondrial fragmentation can occur simultaneously with mitophagosomal biogenesis (Yamashita *et al*, 2016). Indeed, Yamashita and collaborators reported that the machinery responsible for the initiation and elongation of the isolation membrane (including FIP200, ATG14 and WIPIs) is recruited onto tubular mitochondria upon hypoxia or iron chelation and is responsible for mitochondrial fission in a DRP1-independent manner. In addition, in several biological response, most of the mitophagy receptors, including BNIP3L, have been shown to trigger mitochondrial fragmentation as observed in myocytes of high fat diet-fed mouse (da Silva Rosa *et al*, 2021) or during human keratinocyte differentiation (Simpson *et al*, 2021).

  These comments were added to the discussion section of the revised manuscript (lines 301-309).

7. Fig. 7B and E. The suppressive effect of BNIP3L knockdown on *Brucella* reinfection is dramatic, but less so on aBCV formation. Should proportionality not be expected in this case, or would the distinct population of mBCVs also contribute to bacterial egress? This could be partially addressed by establishing whether mBCV formation is BNIP3L-dependent (see major comments).

- Indeed, the difference in the magnitude of the effect of BNIP3L depletion on reinfection foci and aBCV occurrence might be unexpected. What is however important to note is that the exact mechanism leading to aBCV formation and bacterial egress are still poorly understood. We nevertheless agree with the reviewer about the putative role of mBCVs in bacterial egress. However, as shown in Fig. R3 (see above), and as already discussed with the referee #1, BNIP3L depletion does not alter the occurrence of mBCVs, suggesting that mBCVs do not seem to contribute to bacterial egress. These results are now included in the in the revised version of the manuscript and presented in Fig. EV5B-C.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

Verbeke and colleagues present compelling evidence that *Brucella* infection modifies mitochondrial network integrity, affecting BNIP3L-mediated mitophagy to impact pathogen egress. In general, the study is well conducted and presented. The Parkin-independent nature of the phenotype is convincing, and the identification of mitochondrial BCVs is exciting. In general, I am supportive of this manuscript and recommend, for improved clarity, that the findings presented should be discussed further with respect to pre-existing literature.

- We thank the referee for his/her positive opinion of our study. As detailed below, we have addressed all the clarifications raised that have been included in the revised version of the manuscript.

Specific major concerns essential to be addressed to support the conclusions

1. It would be beneficial to consolidate the effects of *Brucella* on mitophagy empirically. Pre-existing evidence exists that *Brucella* infection modifies the autophagy pathway (PMID: 9826346). Moreover, BNIP3L/NIX also act as 'eat-me' signals for peroxisomes to mediate pexophagy (which is also triggered by iron depletion - PMID: 36215693). To address this, the authors might want to consider the following:

a. Infection experiments with macroautophagy vs mitophagy reporters would uncouple these effects (e.g. mCherry-GFP-LC3). This will unambiguously clarify if the mitophagy events observed are attributable to macroautophagy.

- We agree with the referee that some autophagic actors such as ULK1, Beclin and ATG14L are required for proper *Brucella* intracellular cell cycle (Starr *et al*., 2012, *Host Cell Microbe*). However, in this study, the authors showed that LC3 was dispensable for autophagy, suggesting that atypical/non canonical autophagic mechanisms were highjacked by the bacteria. This observation was reinforced by the work of Hamer and colleagues who showed that ATG5 (which is part of the LC3 conjugation complex) is not required for *Brucella* intracellular replication (Hamer *et al*., 2005, *BMC Microbiology*). Whether ATG5 is required for bacterial egress was nevertheless not assessed.

- In addition, we could indeed test the occurrence of macroautophagy in our model using the mCherry-GFP-LC3 reporter, but we are not fully convinced that this experiment will give new insights or will answer the question of the referee. Indeed, since BNIP3L-mediated mitophagy requires LC3 (due to its LC3-interacting region motif) and since *Brucella* triggers LC3 recruitment at the mitochondria (see Fig. 2A-B of the revised manuscript), we could expect and anticipate an increase in acidified mCherry-LC3 in infected cells as well. Moreover, the information from this experiment will not change the message/conclusion that *Brucella* induces mitophagy.

b. Comparative profiling of peroxisomal vs mitochondrial mass (e.g. PMP70/Catalase (peroxisomes) and HSP60 (mitochondrial matrix) immunoblots). However, the reviewer appreciates that bulk protein analysis may not be appropriate if all cells are not infected upon B. abortus treatment. Alternatively, an assessment of peroxisomal network architecture (PMP70 or catalase immunostaining) would prove informative.

- We agree with the referee that studying pexophagy in our model would be of interest since BNIP3L has also been identified as an iron-dependent pexophagy receptor (Wilhelm *et al*, 2022). To answer the question to determine whether a putative BNIP3L-mediated pexophagy response induced by *Brucella* could be observed in infected cells, we first analysed the morphology of peroxisomes using the PEX14 marker in *Brucella*-infected cells. As shown on the Fig. R10 below, we did not observe any difference in peroxisomal morphology in infected cells when compared to non-infected control cells. Moreover, since our study aims to focus on the interplay between *Brucella* and mitochondria, this question raised by the referee would

rather open other new interesting research areas in *Brucella*-organelle crosstalk but might be "out of scope" of our current study.

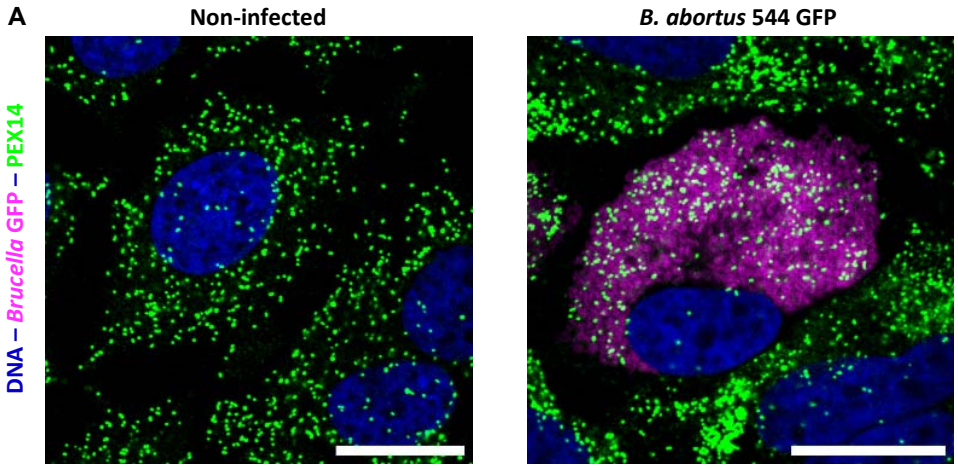

Figure R10. *B. abortus* do not seem to alter peroxisomal morphology.

A. Representative confocal micrographs of HeLa cells infected with *B. abortus* 544 GFP (Magenta) for 48 h, then fixed and immunostained for PEX14 (Alexa Fluor 568 – Green). DNA was stained with Hoechst 33258 (Blue). Scale bars: 20 µm.

2. HIF1-alpha stabilisation occurs rapidly in response to iron depletion (PMID: 32420530), and mitochondrial iron depletion exerts a range of highly specific metabolic changes leading to mitophagy (PMID: 35411952). The authors posit that *Brucella* infection triggers cellular iron depletion. Because mitochondria are a significant source of intracellular iron (PMID: 30485761), could *Brucella* infection deplete mitochondrial iron to launch NIX/BNIP3L and mitophagy? This would be impossible to test but should at least be considered by the authors as a possible mechanism in the revised manuscript. The intimate association between *Brucella* and the iron-rich mitochondrial network would make complete sense and refine assumptions about the precise pool of labile iron affected by the pathogen.

- We thank the reviewer for this relevant comment. This hypothesis is indeed the one we consider to be true and attempted to develop in our discussion. We now put a stronger emphasis on this hypothesis in the manuscript as it might have been only indirectly mentioned and referred to (Lines 328-342).

3. The effects on the mitochondrial network are apparent, but can the authors comment if this phenotype reflects increased mitochondrial fission? Additionally, the authors present a very nice EM image in the paper. Does infection broadly affect the architecture, or are there discernible alterations to mitochondrial ultrastructure? If already performed in their previous article - it would help the reader for the present findings to be placed in the broader context. Can the authors comment in more explicit detail for the reader - is the mitochondrial fragmentation phenotype unique to *Brucella* infection, or is it something that happens during all infection paradigms? Some evolutionary perspectives would also bolster the revised discussion of this phenomenon.

- We again agree with the referee that mitochondria and cristae ultrastructure alteration might be expected in our model, but are not obvious, especially when considering the FIB/SEM results (Fig. 8G and Movie 2 of the revised version of the manuscript) revealing that the ultrastructure of mitochondrial cristae observed in mBCVs look very similar to those of "normal" mitochondria in control non-infected cells.

- Regarding the phenotype of mitochondrial fragmentation, it is clearly not unique to *Brucella*. As mentioned in line 349-353 of the revised manuscript, other intracellular pathogens such as *L. monocytogenes, S. flexneri*, L. *pneumophila*, and *M. tuberculosis* and also trigger mitochondrial fragmentation. The advantage of that for the pathogen could be for example to block mitochondria-mediated apoptosis of the host cell, and/or to inhibit mitochondria-

mediated activation of the inflammasome and subsequent pro-inflammatory response that, otherwise, would contribute to clear the pathogen. Some other pathogens such as *Chlamydiae spp.* rather induce mitochondrial fusion to preserve mitochondrial ATP production required for bacterial survival. A comment about the evolutionary aspects of the manipulation of mitochondria by pathogens was therefore added in the revised manuscript (lines 344-363).

Minor concerns that should be addressed

1. It would be helpful to understand the magnitude of the mitophagy response induced by *Brucella* relative to the canonical iron-depletion NIX/BNIP3-mediated response. An experiment with deferiprone (DFP) included as a positive control would inform the reader as to the extent of mitochondrial turnover in these cells.

- We agree with the referee and as requested, a positive control using another iron chelator, deferoxamine (DFO) was used to compare the magnitude of mitophagy induction by *Brucella* using the FIS1-mCherry-GFP reporter. As shown on the Fig. R11 below, the number of FIS1-mCherry-positive (and GFP-negative) structures are comparable between the *B. abortus*-infected condition and the DFO-treated condition, suggesting that the magnitude of mitophagy induction seems to be similar between these two treatments. This figure is now added in the revised version of the manuscript as an Appendix Fig. S2.

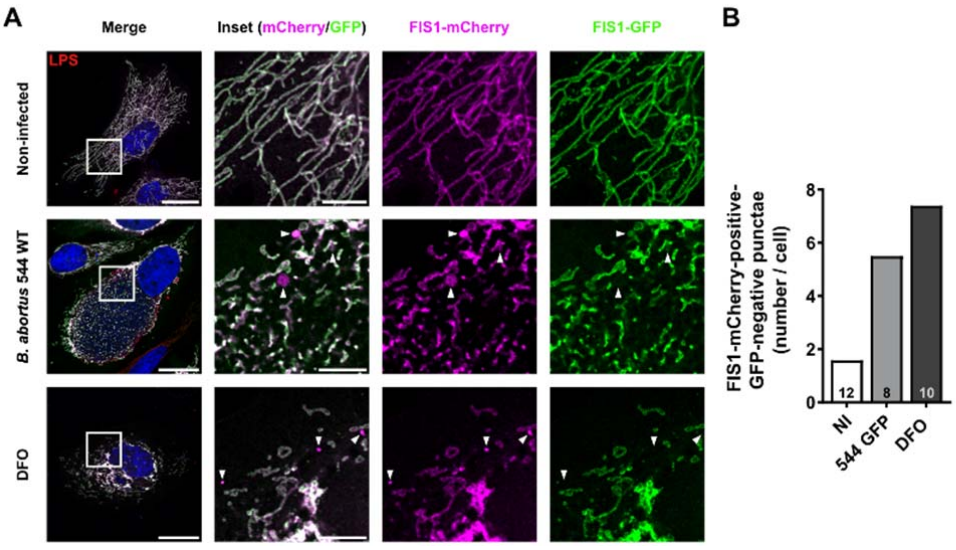

Figure R11. *B. abortus* triggers a similar magnitude of mitophagy induction when compared with a DFO treatment.

A. Representative confocal micrographs of HeLa cells transfected with a FIS1-GFP(Green)-mCherry(Magenta) expression construct, infected or not with *B. abortus* 544 for 48 h, then fixed and immunostained for *B. abortus* LPS (Alexa Fluor 633 – Red). A treatment of DFO (150 µM) for 48 h was used as a positive control. DNA was stained with Hoechst 33258 (Blue). Arrows indicate FIS1-mCherry-positive-GFP-negative punctae. Scale bars: 20 µm. Inset zoom scale bars: 5 µm.
B. Quantification of the number of FIS1-mCherry-positive-GFP-negative punctae per HeLa cell in the indicated conditions from micrographs shown in (A). Data are presented as means from n=1 experiment (the numbers indicated in the columns represent the number of cells analysed per condition).

2. A more comprehensive and precise discussion regarding the presence of *B. abortus* inside TOMM20 membranes should be included in the revised manuscript. Evidence suggests that mitochondria can shed TOMM20-positive vesicles (MDVs, that can be signalling or degradative) and even contribute membrane to autophagosomes in distinct contexts, and PINK1/Parkin-play a role in MDV formation. Because the authors utilised HeLa cells which are devoid of endogenous Parkin, this is likely not the mechanism at play. Nonetheless, how do they get there? In their discussion - can the authors signpost important experimental avenues to dissect this in future studies?

- We thank the referee for this very interesting comment. We now included a paragraph exploring several hypotheses and perspectives about the mechanisms of mitochondrial colonisation by pathogens in the discussion of the revised manuscript (lines 391-412).

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

- In the introduction, it is stated that canonical, ubiquitin-dependent mitophagy depends on the PINK1-Parkin pathway. This assumption is valid only for cell lines and is not supported by the in vivo evidence. The introduction should be updated to reflect the current state of the art, and a distinction should be made between the basal and stress-induced pathways. This would also flow nicely with the Parkin-independent phenotypes reported in their paper.

- We thank the referee for the comment. This paragraph in the introduction was updated given more details about basal and stress-induced mitophagy (lines 101-108). Because our study did not involve *in vivo* experiments, we did not emphasise the mechanisms of mitophagy *in vivo* that are in addition, very poorly described/studied.

- "During mitophagy, targeted mitochondria interact with the autophagy protein LC3 inserted in autophagic phagophores, which are recruited around their cargo" - I suggest the use of 'ATG8-proteins', a term that also captures the GABARAP proteins which can participate in this process.

- We agree with this referee and the term “ATG8 proteins” was used to comprise GABARAP and GABE16 proteins (lines 99-100).

- Several mitophagy reporters exist-Allen et al. utilised mCherry-GFP-FIS1mt101-152 (commonly known as mito-QC). The authors refer to "FIS1-mCherry-GFP". Was mito-QC used, or is this a new FIS1-based reporter? Consistency would be helpful for the naming of the precise reporter used.

- We confirm that the reporter we have used in our study is the same used by Allen and colleagues, also called mito-QC. The nomenclature was adjusted in the material and methods section for better coherence and consistency (line 469).

- Labelling in Figures 4-6 is unconventional (GFP is red, and HIF1-alpha is green etc.). This is not a dealbreaker, but as pixels are pseudo-coloured in any case, it may be helpful for readers if the colours were reassigned to their corresponding conventions in the representative images within the figures.

- We agree with the referee that the choice of pseudo-colours used for some panels are unconventional. However, we opted for the best pseudo-colouring combination in order to optimally highlight our results. As requested in the *EMBO Journal* figure guidelines, for co-immunostaining that aim to highlight organelle morphology or contacts, the choice of green and magenta should be used in priority for the structures of interest, and other colours such as red or blue should be used for second rate structures. Indeed, the use of colours that are suitable for colour-blind readers should be always favoured. For example, the use of magenta/green is preferred over red/green for 2-channel images.

- Thus, for immunofluorescence experiments with 3 or more colours, as the GFP-expressing *Brucella*-related signal is not the first of interest in our study, but so do mitochondria and autophagic markers, we chose to artificially put *Brucella* staining in red, and the structures of interest in green and magenta. As shown in the Fig. R12 below, you might appreciate how TOMM20 and LAMP1 staining are easier to visualise in the right figure (colour combination used in the manuscript) when compared to the left figure (real corresponding colours). Moreover, assigning bacteria in red allows easier visualisation of co-localisation spots between TOMM20 (magenta) and LAMP1 (green) which appear white (right figure) when compared to co-localisation between magenta and red which are almost not visible (left figure).

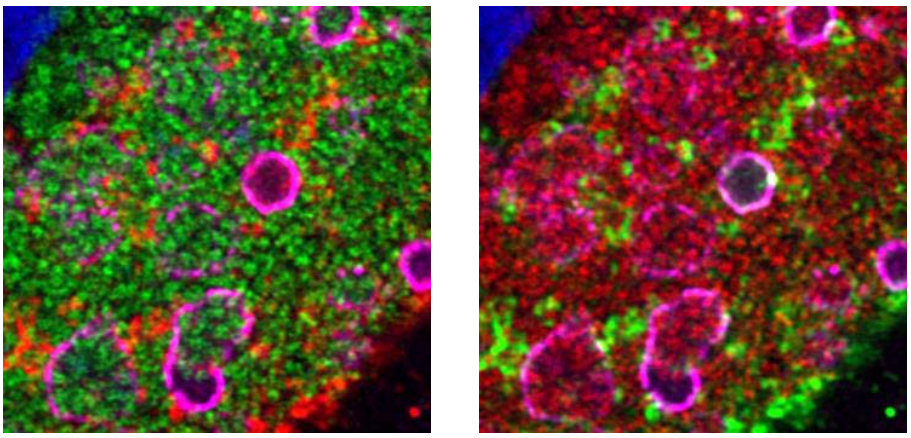

Figure R12. Comparison between "conventional" and "colour-blind friendly" settings for fluorescence microscopy colours.

- A more streamlined figure 8 (step-by-step) may improve clarity for the uninitiated reader (at present, it is aesthetic but takes a while to figure out).

- We thank the referee for the comment that will make the figure easier to understand for the chronology of events. We have now added numbers on the different steps/arrows to clarify the sequential steps highlighted by this summary Fig. 9 in the revised manuscript.

- Violin plots combined with the scatterpoints may be more informative for statistical interpretation of data distribution.

- We agree with the reviewer that violin plots are always preferable for a better view of data distribution. However, this representation of data is only suitable for results coming from experiments with a very high number of independent replicates. In our study, the number of biological replicates (independent experiments) are ranging between 3 and 5 depending on the read out. While the number of events (cells numbers, positive markers,…) analysed is of course higher, that is the reason why we present the data as means +/- SD in the different column charts.

Conclusion:

As a conclusion, and in respect to all referee's comments, criticisms and suggestions, we have made several adjustments to the structure of our manuscript, especially in the order of the presented results. Please find hereafter a summary of the changes we have made in the reorganisation of the figures for the revised manuscript, compared to the first submission. Lines in bold represent new/moved figures.

First Submission

Main figures:

Fig. 1 **HeLa** – Mitochondrial Fragmentation
Fig. 2 **HeLa** – Swollen mitochondria + mBCV
Fig. 3 **HeLa** – Mitophagy (LC3 / FIS1 reporter)
Fig. 4 **HeLa** – HIF-1α / BNIP3L axis activation
Fig. 5 **HeLa** – Iron => HIF-1α / BNIP3L
Fig. 6 **HeLa** – siBNIP3L => Fragmentation / Mitophagy
Fig. 7 **HeLa** – siBNIP3L => Egress / aBCV
HeLa – mBCV different from aBCV
Fig. 8 Summary figure

Supplemental figures:

Fig EV1. **HeLa** – STED (big mBCVs)
Fig EV2. **HeLa** – LC3 details at 24-72 hpi
Fig EV3. **HeLa** – Mitochondrial potential and parkin
Fig EV4. **HeLa** – Hypoxia (EF5)
Fig EV5. **HeLa** – mtROS (MitoSOX / MitoTEMPOL)
Fig EV6. **HeLa** – siBNIP3L efficiency (WB)
Fig EV7. **HeLa** – siBNIP3L => *Brucella* replication

Revised manuscript

Main figures:

Fig. 1 **HeLa** – Mitochondrial Fragmentation
Fig. 2 **HeLa** – Mitophagy (LC3 / FIS1 reporter)
Fig. 3 **HeLa** – HIF-1α / BNIP3L axis activation
Fig. 4 **HeLa** – Iron => HIF-1α / BNIP3L
iBMDM – Iron => BNIP3L
Fig. 5 **HeLa** – siBNIP3L => Fragmentation / Mitophagy
Fig. 6 **HeLa** – siBNIP3L => Egress / aBCV
Fig. 7 HeLa – Iron => Egress / aBCV
iBMDM – Iron => Egress
Fig. 8 HeLa – mBCV (Confocal)
iBMDM – mBCV (Confocal)
HeLa – mBCV (FIB/SEM)
HeLa – mBCV different from aBCV
Fig. 9 Summary figure

Supplemental figures:

- max. 5 Expanded View figures

Fig EV1. **HeLa** – Mitochondrial potential and parkin
Fig EV2. **HeLa** – Hypoxia (EF5)
Fig EV3. **HeLa** – mtROS (MitoSOX / MitoTEMPOL)
Fig EV4. **HeLa** – siBNIP3L => brucella replication
Fig EV5. HeLa – STED (big mBCVs)
HeLa – mBCVs NO BNIP3L
HeLa – mBCVs NO Iron
HeLa – mBCVs NO LC3

- Appendix supplemental figures

Fig S1. **HeLa** – LC3 details at 24-72 hpi
Fig S2. HeLa – FIS1 reporter => Comparison with DFO
Fig S3. **HeLa** – siBNIP3L efficiency (WB)
iBMDM – siBNIP3L efficiency (WB)
Fig S4. HeLa – Swollen mitochondria

References:

Lobet E, Willemart K, Ninane N, Demazy C, Sedzicki J, Lelubre C, De Bolle X, Renard P, Raes M, Dehio C, *et al* (2018) Mitochondrial fragmentation affects neither the sensitivity to TNFα-induced apoptosis of Brucella-infected cells nor the intracellular replication of the bacteria. *Sci Rep* 8: 1–17

da Silva Rosa SC, Martens MD, Field JT, Nguyen L, Kereliuk SM, Hai Y, Chapman D, Diehl-Jones W, Aliani M, West AR, *et al* (2021) BNIP3L/Nix-induced mitochondrial fission, mitophagy, and impaired myocyte glucose uptake are abrogated by PRKA/PKA phosphorylation. *Autophagy* 17: 2257–2272

Simpson CL, Tokito MK, Uppala R, Sarkar MK, Gudjonsson JE & Holzbaur ELF (2021) NIX initiates mitochondrial fragmentation via DRP1 to drive epidermal differentiation. *Cell Rep* 34: 1–18

Starr T, Ng TW, Wehrly TD, Knodler LA & Celli J (2008) Brucella intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic* 9: 678–694

Wilhelm LP, Zapata-Muñoz J, Villarejo-Zori B, Pellegrin S, Freire CM, Toye AM, Boya P & Ganley IG (2022) BNIP3L/NIX regulates both mitophagy and pexophagy. *EMBO J* 41: 1–19

Yamashita SI, Jin X, Furukawa K, Hamasaki M, Nezu A, Otera H, Saigusa T, Yoshimori T, Sakai Y, Mihara K, *et al* (2016) Mitochondrial division occurs concurrently with autophagosome formation but independently of Drp1 during mitophagy. *J Cell Biol* 215: 649–665

Dear Dr. Arnould,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by two of the original referees, who find that their previous concerns have been addressed and now recommend publication of the manuscript. There remain only a couple of minor editorial points that have to be addressed before I can extend formal acceptance of the manuscript:

1. Please move the "Data Availability" section to the end of the "Materials and Methods" section.
2. Figure panel 5E is mentioned in the figure legend and in the manuscript text, but is missing from the figure itself.
3. Figure panels 9A, B are not mentioned in the manuscript text.
4. It appears that figure panels from Figure 3A, lower row, have been reused in figure EV2A, B, C. Please indicate this in the figure legend.
5. CRediT has replaced the traditional author contributions section because it offers a systematic, machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our online submission system to add specific details on the author's contribution. More information is available in our guide to authors.
6. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest)
7. Please remove movie legends from the main manuscript file; each movie legend should be zipped with the corresponding movie file. Please update the nomenclature to Movie EV1, etc.
8. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best regards,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (3rd Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The revised manuscript has significantly improved. The authors have addressed all my concerns/comments and clarified the role of the different phenotypes observed. I have no additional suggestions and I congratulate all authors for this impressive and rigorous work.

Referee #2:

General Summary:
In this revised version of their manuscript, Verbeke and colleagues have made extensive efforts to respond to all reviewers' comments with the inclusion of several new experimental datasets. These revisions satisfactorily address my original concerns and suggestions, in that they clarify the role of iron-dependent BNIP3L-mediated mitophagy in promoting bacterial egress via aBCV formation and extend its relevance to macrophages, while also determining that mBCV formation is not BNIP3L-dependent nor related to bacterial egress. Overall, this is a compelling study with broad impact to the field of microbial pathogenesis.

Minor comment:
A very minor comment I have is that the authors should use past tense sentences in their description of their experimental results (data was obtained in the past when experiments were performed), instead of present tense grammatical constructions, which is more appropriate to conclusions.

The authors have addressed all editorial requests.

Dear Dr. Arnould,

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, I would like to propose a couple of minor changes in the article title, abstract and synopsis. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look at the text below and in the attached manuscript text file and let me know if any corrections are necessary.

Title:
Host cell egress of Brucella abortus requires BNIP3L-mediated mitophagy

Blurb:
ER-replicating intracellular pathogen Brucella abortus relocalizes to mitochondria and induces their fragmentation during late stages of cellular infection.

Synopsis:
The facultative intracellular pathogen Brucella abortus forms a replicative niche in the endoplasmic reticulum and hijacks the autophagic machinery for its egress from the host cell. This study shows that B. abortus induces BNIP3L-mediated mitophagy, which is required for bacterial egress and infection of neighbouring cells.
• B. abortus triggers BNIP3L-mediated mitophagy during the late steps of cellular infection.
• B. abortus induces BNIP3L expression in a manner dependent on iron and HIF-1α stabilization.
• Iron and BNIP3L-mediated mitophagy are required for bacterial egress from host cells.
• B. abortus are found inside mitochondria of HeLa cells and immortalized bone marrow-derived macrophages.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Best regards,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

EMBO Press Author Checklist

Corresponding Author Name: Thierry Arnould
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2022-112817

USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods (Reagent and antibodies section)

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Material and Methods (Mammalian cell culture)
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Material and Methods (Mammalian cell culture)

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Material and Methods (Bacterial strains and culture)

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknoledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Material and Methods (Statistical analyses)

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	**Yes**	Material and Methods (Bacterial strains and culture)
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	**Yes**	Material and Methods (Bacterial strains and culture)
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	**Yes**	Material and Methods (Bacterial strains and culture)

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	