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# SNX3 drives maturation of Borrelia phagosomes by forming a hub for PI(3)P, Rab5a, and galectin-9

Matthias Klose, Johann Salloum, Hannes Gonschior, and Stefan Linder

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*Monitoring Editor: Craig Roy*

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## Transaction Report:

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

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January 30, 2019

Re: JCB manuscript #201812106

Dr. Stefan Linder
Institute for Medical Microbiology, Virology and Hygiene
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Dear Dr. Linder,

Thank you for submitting your manuscript entitled "SNX3 drives maturation of Borrelia phagosomes by forming a hub for PI(3)P, Rab5a, and galectin-9". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while both reviewers find the premise of the study interesting and potentially appropriate for the journal, reviewer #1 feels that more insight into the mechanism(s) by which SNX3 recruits Galactin-9 and how this interaction mediates maturation of the Borellia phagosome is needed - and we agree with this assessment. In addition, both reviewers raise a number of other concerns which would need to be addressed in full before the paper would be deemed suitable for publication in JCB.

If you wish, you might consider formulating a revision plan indicating how you would propose addressing these concerns so that we can give you some editorial feedback before you proceed with the revisions.

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

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

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

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

We hope that the comments below will prove constructive as your work progresses.

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

Sincerely,

Craig Roy, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
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Reviewer #1 (Comments to the Authors (Required)):

In this manuscript Klose and Linder analyzed the molecular basis of the compaction and maturation of Borrelia-containing phagosomes in human macrophages. Proper compaction and maturation are required for a successful microbicidal response. The authors report that SNX3, which is recruited by PI(3)P, is required for these processes. More interestingly, using truncation analysis they report that the C-terminal domain of SNX3 is essential and that it operates, at least in part, by recruiting galectin-9. They proceeded to show that galectin-9 is itself required for compaction and that it functions downstream of SNX3.

While novel and interesting, these observations seem preliminary to the extent that the mechanism of action of galectin-9 is not resolved. How does it promote the recruitment of Rab5? Is its lectin activity involved? Does it recruit additional proteins that, for some reason, may not have appeared in the mass spectrometry analysis? Does it promote tethering, fusion or acts by some other mechanism?

It is also unclear how SNX3 itself is recruited, since PI(3)P formation by PI3K in endosomes is generally thought to require Rab5, yet in the authors' scheme Rab5 is acquired at a later stage. What is the source of the PI(3)P?

Other comments:
1) What is the evidence that SNX3 is recruited to sites of high curvature? The resolution of conventional confocal microscopy and of the images provided is insufficient to detect the small curvature that BAR domains prefer.
2) Overall degradation of DQ-BSA was unaffected by silencing SNX3, implying that normal endosome maturation is unaffected. Is there any evidence that phagosome maturation is different from endosome to lysosome maturation? Is this a feature of phagosomes or an effect induced by Borrelia and, if so, are the effectors involved known?
3) It is remarkable that the Akt-PH domain was not found where the authors find PI(3,4)P2, since this construct binds PI(3,4)P2 as well as PI(3,4,5)P3. How is this apparent discrepancy explained?
4) How is PI(3)P kept in spots, prevented from diffusing throughout the vacuolar membrane? Can the authors resolve unambiguously PI(3)P present in the vacuole from PI(3)P in adherent vesicles?
5) What is the process underlying "compaction"? Is it caused by removal of membrane from the long spiral vacuoles or by application of a mechanical force? Defining the basis of compaction is important to understand the role of SNX3 and of galectin-9.
6) Does siRNA to galectin-9 prevent delivery of DQ-BSA to lysosomes?
7) Why was the retromer complex known to associate with SNX3 not found among the immunoprecipitation-mass spec candidates?
8) How can galectin-9 co-precipitate with SNX3 if it seems to be distributed (largely) in a separate vesicular population? What targets it to those other vesicles?

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

The manuscript by Klose and Linder entitled "SNX3 drives maturation of Borrelia phagosomes by forming a hub for 2 PI(3)P, Rab5a, and galectin-9" explores the role of the sorting nexin SNX3 in the formation of the B. burgdorferi phagosome in conjunction with the early endosomal GTPase Rab5 and galectin-9. The studies show that the role of SNX3, in the Rab5 vesicle, is to mediate early endosome and phagosomal fusion by binding PI(3)P on the phagosomal coat. SNX3 subsequently recruits and binds galectin-9 found in separate vesicles and contributes to the process of early phagosome maturation. These studies further our understanding of the early events in B. burgdorferi phagosome formation.

Overall this is a solid manuscript with novelty regarding early phagosomal formation of Borrelia. The role of SNX3 and Gal9 in B. burgdorferi phagosomes has not been previously described. Beyond B. burgdorferi the novelty is fairly low as the PX domain of SNX3 and binding to PI(3)P has been previously described as well as the role of Gal9 in phagosomes. Nevertheless, this paper puts all these components together into a clearer picture of the biology of early phagosome formation and specifically for B. burgdorferi and is well suited for publication at JCB.

Specific comments:

1) Material and Methods - A better description of the microscopy quantification methods (programs used, parameters etc.) and image analysis would be helpful. Including more details as to how measurements such as phagosomal volume were determined.

2) Fig. 2. - Staining of Borrelia in Fig. 2 with Anti-Borrelia antibodies would be more convincing than using a DNA dye like Hoechst33342 to detect the spirochetes.

3) Fig. 7. - The localization of Gal9 with Borrelia and the decrease in localization by microscopy is not particularly convincing. The authors should make sure that both the control siRNA and the snx3 siRNA images are zoomed in equally for better evaluation of the presence of Gal9 on B. burgdorferi. While there might be mathematically statistical significance in the decrease of Gal9 in the snx3 siRNA, the majority of events show very similar fluorescence intensity to the controls and the data looks like it is skewed towards statistical significance due to very few events in the control. An improved staining protocol for endogenous detection with anti-Gal9 or over-expression might be more convincing in this particular figure.

Dear Dr. Roy,
dear Dr. Spencer,

we are submitting a revised version of our manuscript entitled "SNX3 drives maturation of *Borrelia* phagosomes by forming a hub for PI(3)P, Rab5a, and galectin-9", and ask you to consider its suitability for *Journal of Cell Biology*. We are very grateful for the reviewers´ constructive comments and also for your editorial guidance and have tried to address the raised points as closely as possible. Moreover, based on their help in revising the paper, Johann Salloum and Hannes Gonschior have been added as coauthors. Please find a point-by-point response below. The respective changes are marked in red in the manuscript.

**Reviewer #1**

In this manuscript Klose and Linder analyzed the molecular basis of the compaction and maturation of Borrelia-containing phagosomes in human macrophages. Proper compaction and maturation are required for a successful microbicidal response. The authors report that SNX3, which is recruited by PI(3)P, is required for these processes. More interestingly, using truncation analysis they report that the C-terminal domain of SNX3 is essential and that it operates, at least in part, by recruiting galectin-9. They proceeded to show that galectin-9 is itself required for compaction and that it functions downstream of SNX3. While novel and interesting, these observations seem preliminary to the extent that the mechanism of action of galectin-9 is not resolved.

Thank you for the constructive comments, they are much appreciated.

1) *How does it (galectin-9) promote the recruitment of Rab5?*
Galectin-9 is not involved in the recruitment of Rab5a. Rab5a is transported on SNX3-positive vesicles (Suppl. Fig. 2I). This vesicle population is distinct from the one carrying galectin-9 (lines 398-399 in revised version: "Collectively, the data indicated that SNX3 enables the contact of two distinct vesicle populations, carrying either Rab5a or galectin-9…").

2) *Is its lectin activity involved?*
Galectin-9 features a short N-terminal region and two carbohydrate binding domains (CRD1, CRD2), which are bridged by a linker region. Both CRDs differ in their affinities for specific carbohydrates. Accordingly, several critical amino acid residues have been identified that are necessary for binding of specific carbohydrates, including Ala46 for the binding of Forssman pentasaccharides (Nagae et al., JMB, 2008), Asn137 for poly-N-acetyllactosamine (Nagae et al., Glycobiol., 2009) and Arg221 for sialylated oligosaccharides (Yoshida et al., JBC, 2010), the latter corresponding to Arg252 in our paper, due to a shorter linker region used in (Yoshida et al., 2010).
We generated respective siRNA-insensitive constructs and tested them for their ability to rescue regular phagosomal compaction levels. All of the mutants showed intermediate values for phagosome compaction rescue, which were also not significantly different from the negative control. It is thus unlikely that binding of the specific carbohydrates mentioned is a decisive factor for galectin-9 dependent regulation of phagosome compaction. Still, we can not rule out that binding of other carbohydrates, and especially of carbohydrate side chains of glycosoylated proteins, by galectin-9 plays a role in this process. These results are now shown in the new Fig. 8C,D, and are described in the results section (pp.12,13) and discussion section (p.17). The above mentioned citations have been added to the reference list.

3) *Does it recruit additional proteins that, for some reason, may not have appeared in the mass spectrometry analysis?*
To address this question, we have now performed anti-GFP immunoprecipitations using full length GFP-galectin-9 and analyzed the precipitates by mass spectrometry. Potential candidates were evaluated for their presence at galectin-9 vesicles in immunofluorescence. Promising candidates included RabGTPases such as Rab8a, Rab10 and Rab18, vesicle regulators of the flotillin family, flotillin-1 and -2, and the vesicle docking protein SNAP23. Respective GFP- or mCherry-labeled constructs were expressed in borreliae-infected cells, which were also stained for galectin-9. Further RabGTPases, including Rab4a, Rab6a, Rab7, Rab11, Rab14, Rab21a, Rab22a, Rab27a, and Rab43 were also included in this analysis, to potentially identify the trafficking pathways of galectin-9 vesicles. We found enrichments of Rab6a, Rab8a and Rab21 at borreliae phagosomes, but no specific association with galectin-9 vesicles. By contrast, we detected prominent colocalization of flotillin-2 with galectin-9 at vesicles in both fixed and live specimens. These results are now shown in the new Suppl. Fig. S7A-Q and in the new Fig. 7I.

4) *Does it promote tethering, fusion or acts by some other mechanism?*
We now show that galectin-9 vesicles are also positive for flotillin-2, a member of the flotillin family that is regulating membrane and protein recycling pathways (Meister and Tikkanen, Membranes, 2014), for example in association with Rab11a- and SNX4-positive recycling compartment in HeLa (Solis et al., 2013), with a Rab7-positive endosomal recycling compartment in MDA-MB-231 (Planchon et al., JCS, 2018), or with galectin-3-positive endosomes in MDCK cells (Straube et al., Traffic, 2013). Taken together, these findings seem to point to a connection between members of the galectin and flotillin, but also of the Rab and SNX families, in the control of endosomal recycling pathways, with the individual family members involved potentially depending on the specific cellular context. It is thus likely that galectin-9 exerts its influence on borreliae phagosome compaction by recruiting flotillin-2. These findings are now mentioned in the Discussion section (pp.18,19). As flotillins are emerging as the master switches of these recycling processes, through multiple interactions with regulators such as CAP, Crk or TC10 (Stürmer, Trends Cell Biol., 2009), we believe that the exact clarification of the galectin-9-/flotillin-dependent regulation of phagosome compaction is outside of the scope of this revision. We hope that the reviewer agrees with us on this.

5) *It is also unclear how SNX3 itself is recruited, since PI(3)P formation by PI3K in endosomes is generally thought to require Rab5, yet in the authors' scheme Rab5 is acquired at a later stage. What is the source of the PI(3)P?*
Based on our previous results (Naj and Linder, Cell Rep, 2015), we are very sure that borreliae-containing phagosomes are decorated by Rab22a and are only at a later stage contacted by Rab5a-positive vesicles. However, it is still conceivable that vesicular Rab5a comes into contact with the phagosome surface and thus contributes to PI(3)P generation at phagosomes. In addition, our experiments using the PI3 kinase inhibitor Wortmannin that led to dispersed localization of the PI(3)P sensor (Fig. 3O-Q) indicate that PI(3)P at phagosomes and endosomes is likely generated by phosphorylation of inositides, and not by dephosphorylation of phosphoinositides, e.g. PI(3,4) $P_2$.
In order to determine whether PI(3)P is derived from the cell surface or from endosomes, or is generated at phagosomes, we have expressed the PI(3)P sensor in borreliae-infected cells and analysed its dynamic localization by live cell imaging. We find that, consistently with the images from fixed samples (Fig. 3E-H), PI(3)P is not enriched at the cell surface, but mostly present at vesicles/endosomes and on phagosomes. In order to determine the source of PI(3)P at borreliae phagosomes, live cell imaging was performed using macrophages expressing the

PI(3)P sensor, either alone, or in combination with the endosomal marker RFP-Rab5a. Interestingly, we observed a gradual accumulation of PI(3)P at borreliae phagosomes, with only occasional contact by PI(3)P-/Rab5a-positive endosomes. Taken together, these observations point to the local generation of PI(3)P at phagosomes, with endosomal delivery of PI(3)P possibly as a contributing source. These results are now presented in the new Suppl. Figure S5 and in the new Suppl. videos 4 and 5 and are mentioned in both the Results (pp.8,9) and Discussion sections (pp.15,16).

Other comments:

6) *What is the evidence that SNX3 is recruited to sites of high curvature? The resolution of conventional confocal microscopy and of the images provided is insufficient to detect the small curvature that BAR domains prefer.*

The reviewer is, in principle, correct, but we would like to point out that SNX3 does not contain a BAR domain and consists mainly of a PX domain that binds PI(3)P, with N-and C-terminal extensions (see Figure 1). Curvature on a level that is detectable by BAR domains is thus probably not involved. We hypothesize that SNX3 is recruited to sites of high curvature, on the level of spirochete/phagosome morphology, by the presence of phagosomal PI(3)P at these sites. We consistently find that SNX3 vesicles are recruited to sites of high curvature of borreliae containing phagosomes. We are providing a gallery of respective images at high magnification for the reviewer to illustrate this point (Figure 1 for referee).

7) *Overall degradation of DQ-BSA was unaffected by silencing SNX3, implying that normal endosome maturation is unaffected. Is there any evidence that phagosome maturation is different from endosome to lysosome maturation? Is this a feature of phagosomes or an effect induced by Borrelia and, if so, are the effectors involved known?*

Phagosome maturation can indeed be affected by internalized microorganisms or their surface-derived substances such as LPS or mannan (reviewed in Pauwels et al., Trends Immunol., 2017). Interestingly, a recent publication on the proteome of latex bead phagosomes from DCs stimulated with LPS vs. non-stimulated DCs showed numerous differences in the recruitment of cellular factors, including SNX3 (Pauwels et al., Mol Cell Proteom, 2019). While *Borrelia* does not contain LPS, the mentioned paper and also previous studies still show that host cell factors can be differentially recruited to phagosomes and that this recruitment is altered upon uptake of different phagocytic cargo.

8) *It is remarkable that the Akt-PH domain was not found where the authors find PI(3,4)P2, since this construct binds PI(3,4)P2 as well as PI(3,4,5)P3. How is this apparent discrepancy explained?*

Thank you for pointing out this discrepancy. We have now prepared a new batch of the Akt-PH domain from frozen stock and expressed it in macrophages. Using this new batch, we indeed find localization of the sensor at borreliae phagosomes. We apologize for this mistake and have now exchanged the respective panels from the supplementary material (Figure S4 E-H) with new representative images. We also added “PI(3,4)P2” in addition to “PI(3,4,5)P3” as a label for the panels.

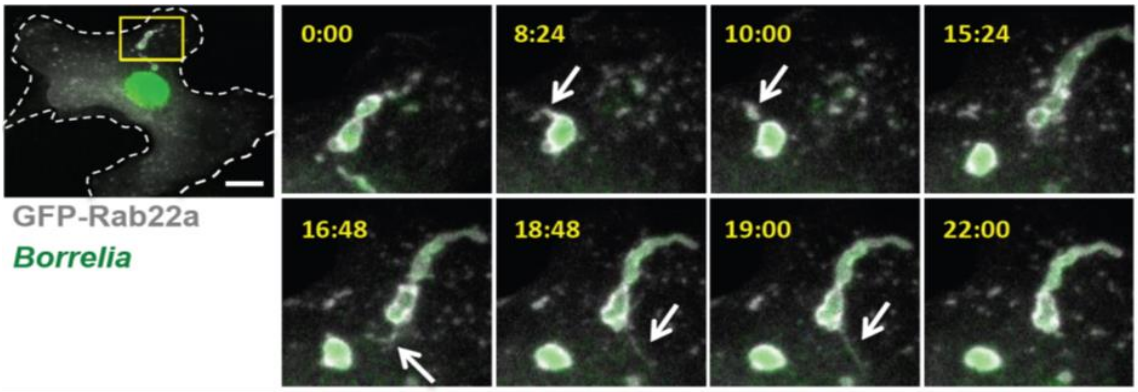
9) *How is PI(3)P kept in spots, prevented from diffusing throughout the vacuolar membrane?*

Apparently, there is a diffusion barrier that hinders free mixing of lipids in the phagosomal membrane. We hypothesize that transmembrane proteins could help to establish microdomains by blocking or reducing diffusion, analogous to the role of integrins described as diffusion barriers in the nascent phagocytic cup (Freeman et al., Cell, 2016). There is a wide variety of potential candidates such CD44 or CD45, which could fulfill this role. To systematically investigate this, identification of potential candidates by purification of

borreliae-containing phagosomes with subsequent mass spectrometry and testing of individual candidates by localization to phagosomes and siRNA-mediated depletion would be necessary. This appears to be beyond the scope of the current manuscript.

10) *Can the authors resolve unambiguously PI(3)P present in the vacuole from PI(3)P in adherent vesicles?*
We can distinguish PI(3)P at phagosomes from vesicular PI(3)P by staining or overexpression of the respective phagosome marker Rab22a and vesicle marker Rab5a. While not all cases can thus be resolved unambiguously, the large majority of cases (~ 80-90%) allows a clear distinction. We are providing a set of respective images, with macrophages expressing the PI(3)P sensor p40phox-GFP, and stained for Rab5a or Rab22a, in both confocal and STED mode to illustrate this point (Figure 2 for referee).

11) *What is the process underlying "compaction"? Is it caused by removal of membrane from the long spiral vacuoles or by application of a mechanical force? Defining the basis of compaction is important to understand the role of SNX3 and of galectin-9.*
Compaction is caused by the formation and removal of membrane tubules from borreliae-containing phagosomes. This process is described in (Naj and Linder, Cell Rep., 2015) and mentioned in the introduction (lines 78-79) of the current manuscript ("…leading to the formation of membrane tubules, which results in successive reduction of the phagosomal surface and compaction of spirochetes.").
The following panel from (Naj and Linder, 2015; Fig. 2) shows two borreliae being compacted in Rab22-positive phagosomes, with the arrows pointing to sites of membrane tubule formation and removal.




12) *Does siRNA to galectin-9 prevent delivery of DQ-BSA to lysosomes?*
We have now performed DQ-BSA measurements of borreliae-containing phagosomes using galectin-9 siRNA, analogous to the experiments using SNX3 siRNA (Fig. 2C-J) (see also reviewer 2, point 2). Using two independent siRNAs, we find highly significant reductions in the proteolytic capacity of borreliae-containing phagosomes, compared to controls. In addition, the overall cellular DQ-BSA-based fluorescence intensity was unchanged. These results are now presented in the new Fig. 9.
For clarification, please note that this experiment measures the proteolytic capacity of phagosomes, based on the de-quenched fluorescence of DQ-BSA within phagosomes, but not the delivery of DQ-BSA to phagosomes.

13) *Why was the retromer complex known to associate with SNX3 not found among the immunoprecipitation-mass spec candidates?*

The immunoprecipitation/mass spec analysis was performed for the GFP-fusion of the SNX3 C-terminal tail (11 aa residues), also named GFP-SNX3-C (lines 305-307 in revised version: "…GFP-SNX3-C was expressed in macrophages, anti-GFP immunoprecipitation was performed, and the resulting precipitate was analysed by mass spectrometry"). In contrast, the retromer complex binds to the N-terminal region of SNX3 (lines 487-488 in revised version: " …the SNX3 N-terminal region has been shown to bind retromer complex (Lucas et al., 2016)").
Please find below a figure illustrating this point. Lucas et al modeled SNX3 (blue) together with the retromer subunits VPS26 (beige) and VPS35 (red). Retromer binding is mediated by the SNX3 N-terminus and the adjacent alpha-helical region. Please note that the C-terminus, which we used for the mass spec analysis, is not part of this interaction.


14) *How can galectin-9 co-precipitate with SNX3 if it seems to be distributed (largely) in a separate vesicular population?*
Please note that only a small subfraction of the total cellular pool of galectin-9 is coprecipitated with GFP-SNX3-C (Fig. 6A: SNX3-C lanes: loading control ("ld") versus eluate ("el")). This subfraction is most probably the one that is binding SNX3 at borreliae phagosomes. We have now added the following sentence to the Discussion (p.17): "Of note, only a small subfraction of the total cellular pool of galectin-9 coprecipitated with the SNX3 C-terminus (Fig. 6A), most probably representing the fraction of galectin-9 that is binding SNX3 at borreliae phagosomes".

15) *What targets it to those other vesicles?*
We have now visualized the siRNA-insensitive galectin-9 constructs in macrophages depleted for endogenous galectin-9. From all constructs tested, only the ΔN construct showed a fully vesicular localization, comparable to the wild type. The N+CRD1 construct showed a mixture of dispersed and vesicular localization, while all other constructs showed dispersed localization. While we can not rule out that some of the truncation constructs may exhibit aberrant folding, this points to a requirement for both CRD domains to target galectin-9 to vesicles. Moreover, the vesicular localization of galectin-9 constructs closely correlates with their respective ability to act in phagosomal compaction. These observations are now mentioned in the results section. Respective images are shown in the new Suppl. Fig. S7R-V.

**Reviewer #2**
The manuscript by Klose and Linder entitled "SNX3 drives maturation of Borrelia phagosomes by forming a hub for 2 PI(3)P, Rab5a, and galectin-9" explores the role of the

sorting nexin SNX3 in the formation of the B. burgdorferi phagosome in conjunction with the early endosomal GTPase Rab5 and galectin-9. The studies show that the role of SNX3, in the Rab5 vesicle, is to mediate early endosome and phagosomal fusion by binding PI(3)P on the phagosomal coat. SNX3 subsequently recruits and binds galectin-9 found in separate vesicles and contributes to the process of early phagosome maturation. These studies further our understanding of the early events in B. burgdorferi phagosome formation.
Overall this is a solid manuscript with novelty regarding early phagosomal formation of Borrelia. The role of SNX3 and Gal9 in B. burgdorferi phagosomes has not been previously described. Beyond B. burgdorferi the novelty is fairly low as the PX domain of SNX3 and binding to PI(3)P has been previously described as well as the role of Gal9 in phagosomes. Nevertheless, this paper puts all these components together into a clearer picture of the biology of early phagosome formation and specifically for B. burgdorferi and is well suited for publication at JCB.

We thank the reviewer for their positive and constructive comments. Still, we would like to point out that both the described role of the SNX3 C-terminal region is novel, as is the role of galectin-9 in phagosome compaction and also its association with phagosomes and its presence at intracellular vesicles in general.
The referee is probably referring to the well-known role of galectin-3, which is recruited from a cytoplasmic pool to the inner leaflet of damaged phagosomes, thus functioning as a reporter of phagosome rupture (Paz et al., Cell Microbiol, 2010; Ehsani et al., Infect Immun, 2012). In contrast, we show that galectin-9 is constituively present at intracellular vesicles in macrophages, and that it regulates the compaction of intact phagosomes, thus driving their maturation and borreliae degradation. To clarify this point, a respective paragraph was added to the Discussion (p. 18), and the two citations have been added to the reference list.

Specific comments:

1) *Material and Methods - A better description of the microscopy quantification methods (programs used, parameters etc.) and image analysis would be helpful. Including more details as to how measurements such as phagosomal volume were determined.*
We agree with the reviewer and have added the requested details to the Materials and Methods section.

2) *Fig. 2. - Staining of Borrelia in Fig. 2 with Anti-Borrelia antibodies would be more convincing than using a DNA dye like Hoechst33342 to detect the spirochetes.*
We agree with the reviewer. The anti-*Borrelia* antibody we normally use gave a high background in DQ-BSA assays. We thus performed the DQ-BSA experiments using GFP-expressing borreliae, for both SNX3- and galectin-9 siRNA treated cells (see also reviewer 1, point 11). In both cases, we find (highly) significant reductions compared to controls. The new results for SNX3-specific siRNAs now replace the previous panels in Fig. 2C-J, while the data gained using galectin-9-specific siRNA are now shown in the new Fig. 9.

3) *Fig. 7. - The localization of Gal9 with Borrelia and the decrease in localization by microscopy is not particularly convincing. The authors should make sure that both the control siRNA and the snx3 siRNA images are zoomed in equally for better evaluation of the presence of Gal9 on B. burgdorferi. While there might be mathematically statistical significance in the decrease of Gal9 in the snx3 siRNA, the majority of events show very similar fluorescence intensity to the controls and the data looks like it is skewed towards statistical significance due to very few events in the control. An improved staining protocol for endogenous detection with anti-Gal9 or over-expression might be more convincing in this particular figure.*

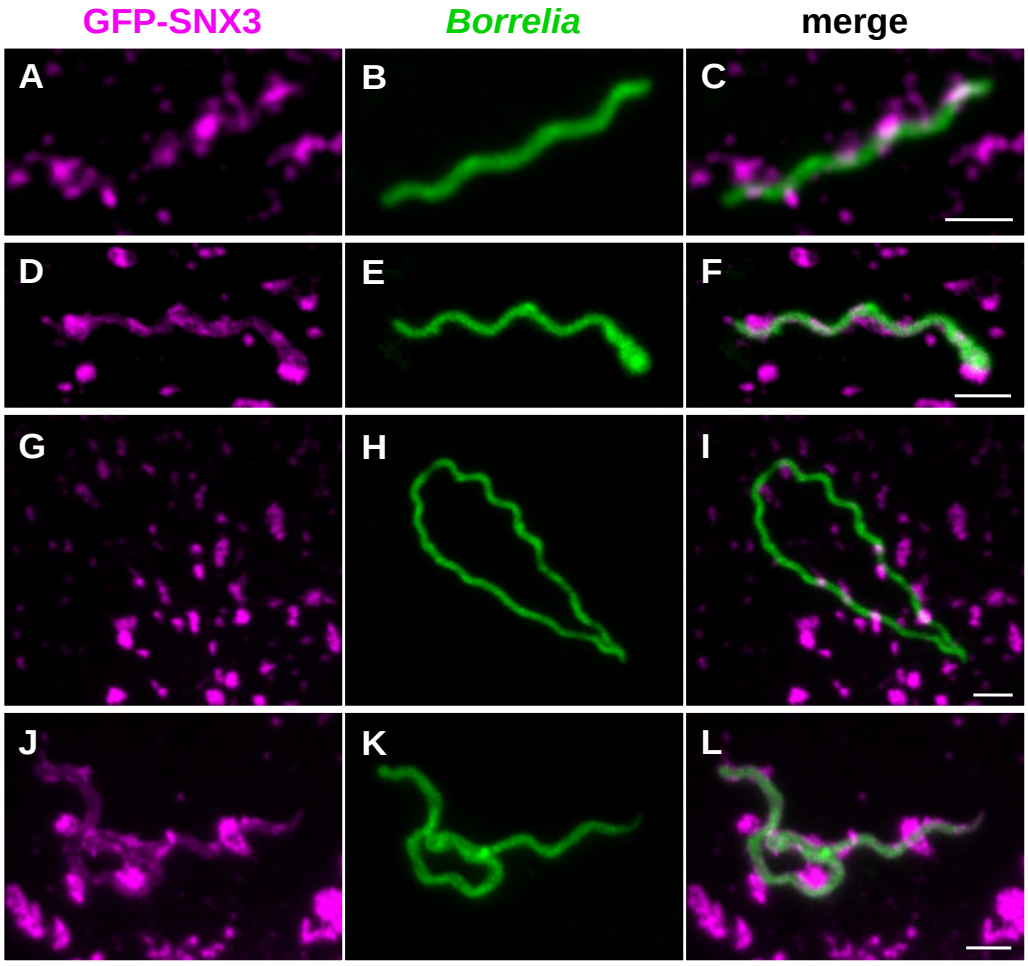
We agree with the reviewer and are now presenting new images for the SNX3 siRNA experiments with equal zoom, compared to controls (new Fig. 9B, B1-B4).
We also agree that the extreme outliers in the Gal9 fluorescence intensity of SNX3 siRNA treated cells (new Fig. 9D), especially in the control, which may lead to a skewed analysis. Therefore, we have performed an outlier labeling analysis, as described in (Hoaglin et al., 1986, Journal of American Statistical Association). This analysis identifies outlier values using the lower and upper quartiles $F_L$ and $F_U$, according to the formula $F_U + 1.5(F_U - F_L)$. According to this analysis, treatment with SNX3 siRNA leads to a value of 1685 a.u. ± 144.5 a.u., compared to 2837 a.u. ± 291.2 a.u., for controls which is significantly different with a *P* value <0.01. The outlier analysis is now described in Materials and Methods, and the respective citation has been added to the reference list.

We are grateful for the time and energy that the referees have invested in the review of our manuscript. We hope that the current version is now suitable for publication in *Journal of Cell Biology*.

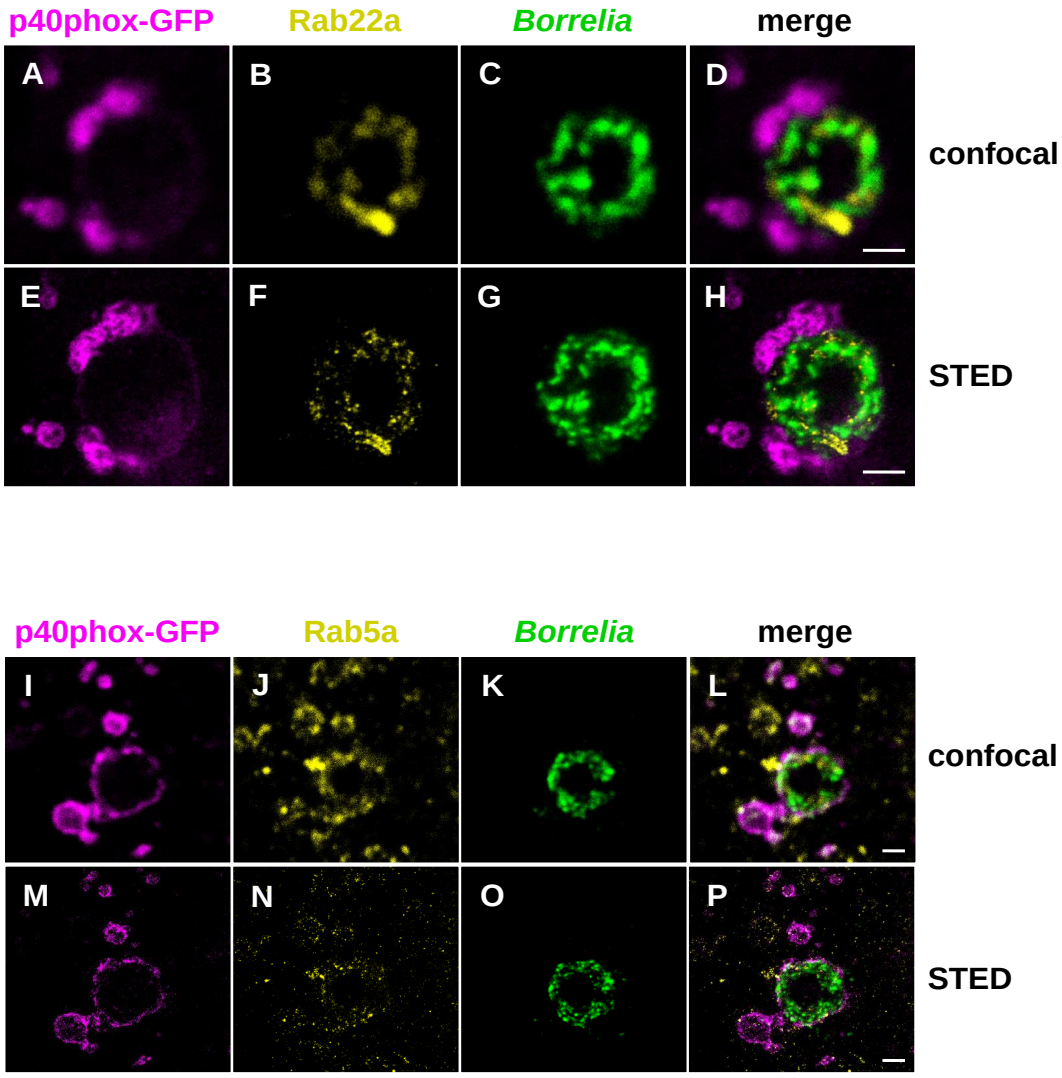
Kind regards,

Stefan Linder and Matthias Klose, on behalf of all coauthors

**Figure 1 for referee**




**Sorting nexin 3 predominantly localizes to sites of high curvature at *Borrelia*-containing phagosomes.** Confocal micrographs of macrophages (insets) expressing GFP-SNX3 (A,D,G,J) with internalized borreliae stained by specific antibody (B,E,H,K), and respective merges (C,F,I,L). Scale bar: 2 µm.




**Differentiation of PI(3)P localized at borreliae phagosomes from PI(3)P localized at vesicles.** Confocal and super resolution micrographs of specimens showing primary macrophages overexpressing PI(3)P-sensor p40phox-GFP, stained for GFP by using anti-GFP nanobodies 647N (A,E,I,M) , RabGTPases Rab22a (B,F) and Rab5a (J,N) stained by rabbit anti-Rab22a/Rab5a and anti-rabbit-AlexaFluor594 antibodies and borreliae stained by mouse anti-*Borrelia burgdorferi* and anti-mouse-ATTO542 (C,G,K,O), respectively, with merges (D,H,L,P).
Scale bar: 1 µm.

June 6, 2019

RE: JCB Manuscript #201812106R

Dr. Stefan Linder
Institute for Medical Microbiology, Virology and Hygiene
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Dear Dr. Linder:

Thank you for submitting your revised manuscript entitled "SNX3 drives maturation of Borrelia phagosomes by forming a hub for PI(3)P, Rab5a, and galectin-9".
Your paper has now been seen by the original reviewers and, as you will see below, reviewer#2 is satisfied with the revisions and recommends publication. However, reviewer#1 still has a number of issues with the manuscript. Although we agree with this reviewer that these points are substantive and addressing them would undoubtedly increase the impact of your paper, we also feel that addressing them experimentally is beyond the scope of the current study. However, we would like for you to address these issues through further discussion. In addition to the other issues raised by the reviewer, we would like for you to comment further on possible retromer-independent roles for SNX3.

Once these issues are addressed, we would be happy to publish your paper in JCB, pending further final revisions necessary to meet our formatting guidelines (see details below).

**Please also be sure to include a point-by-point rebuttal to reviewer #1's comments along with your final revised manuscript.**

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures. You are currently at this limit but please bear it in mind when revising.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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

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

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

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

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

9) Supplemental materials: There are generally strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you currently have 7 supplemental figures. In this case, we should be able to give you the extra space but please try not to increase this number if at all possible.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements

regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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

B. FINAL FILES:

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

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

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

Sincerely,

Craig Roy, PhD
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JCB

Tim Spencer, PhD

Deputy Editor
Journal of Cell Biology

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

The revised manuscript by Klose et al. now includes additional data regarding the possible mode of action of galectin-9 and partially addresses the chronological sequence of events leading to its recruitment to the vacuole. Using mass spectrometry, the authors now propose that flotillin may be a contributor to the compaction of the Borrelia phagosome. In addition, using mutagenesis, they explore the domains of galectin involved in its role in compaction and conclude that the sugar-binding (lectin) domains are unlikely to be critical for its function.

While identification of galectin-9 as a participant in membrane remodeling is very novel and potentially important, some important questions remain unresolved in the present version:

1) Despite the mutagenesis analysis, which rules out sugar binding as an important component, the mode of action of galectin-9 remains obscure. How does it facilitate the removal of excess membrane from the vacuole?
2) The finding that flotillin associates with galectin is interesting and suggestive, but unfortunately the authors did not define how flotillin contributes to compaction or even whether it is actually required for the process.
3) Because SNX3 was reported to associate with the retromer and this complex has been implicated in endosomal tubulation and scission, it is surprising that the authors did not investigate the possible role of retromer in phagosome compaction. The retromer would seem to be ideally poised to remove membrane from the vacuole.
4) The authors believe that Rab5 recruitment is downstream of the acquisition of PI3P, while most of the existing literature attributes the formation of PI3P to recruitment and activation of class III PI3K by Rab5, i.e. the reverse chronological sequence. Validation of the authors' model would seem to require demonstration that satisfactory silencing or inhibition of Rab5 has no effect on the acquisition of PI3P by the Borrelia phagosomal vacuole.

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

This is a re-submitted manuscript by Klose and Linder entitled "SNX3 drives maturation of Borrelia phagosomes by forming a hub for 2 PI(3)P, Rab5a, and galectin-9". The authors have adequately addressed all the previous criticisms and the manuscript and data are much improved. No further modifications are required.