EMBO reports

Rabaptin5 targets autophagy to damaged endosomes and Salmonella vacuoles via FIP200 and ATG16L1

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Dear Prof. Spiess

Thank you for the submission of your research manuscript to EMBO reports. It has been reviewed by the journal-independent platform Review Commons and you have submitted a point-by-point response and outlined a revision plan.

We agree that your study is potentially a nice contribution to EMBO reports and feel that the proposed revision plan will strengthen your data. It will be particularly important to provide further evidence that CQ and monensin induce damage to early endosomal membranes, and to discriminate between canonical and non-canonical autophagy. In this regard it could be useful to indeed analyse the localization of ATG16L1a with Rabaptin5 or the transferrin receptor upon CQ treatment. I also noticed that you have not specifically commented on the concern regarding the 30 minutes CQ treatment from referee 3. I think it would be beneficial to document the effect of short (30 min) vs long (5 hours) treatment regarding lysosomal function (pH, fusion). Regarding the color in Figure 2, I think either way should be OK (greyscale or color), whatever gives better contrast. You could change the merged images to cyan and magenta though to allow color-blind people to see the overlap.

We invite you to submit your manuscript within three months of a request for revision. This would be September 15th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

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7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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The following points MUST be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
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Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

Response/revision plan

General response

The main criticism of the reviewers concerns the distinction between chloroquine (CQ)-induced canonical autophagy and previously characterized LAP-like autophagy. While canonical autophagy can be triggered by membrane damage and subsequent recruitment of galectins and ubiquitination, and involves the ULK complex, Vps34/PI3P, and WIPI2 upstream of the LC3 lipidation machinery (including ATG16L1), LAP-like autophagy was described in particular for entotic vacuoles or latex bead-containing phagophores, where CQ and monensin (Mon) were shown to induce direct recruitment of LC3 to the intact single membrane of the phagosome in a manner independent of ATG13 (and thus the ULK complex), of PI3P production, and of WIPI2 (Florey et al., 2015; Jacquin et al., 2017; Fletcher et al., 2018).

In our study, we argue that Rabaptin5, a bona fide marker of Rab5- and transferrin receptor-positive early endosomes, is involved in autophagy of endosomes that were damaged by CQ or Mon treatment to become positive for ubiquitin and galectin3. We found WIPI2 colocalization with Rabaptin5-positive endosomes within 30 min and a strong increase of WIPI2 and LC3 puncta at longer times. In particular the WIPI2 puncta formation strongly depended on the ULK complex (ULK1/2, ATG13, FIP200), and on Rabaptin5 (which we initially discovered to interact with FIP200). From this, we concluded that the effect of CQ or Mon on endosomes is different from LAP-like autophagy of entotic vacuoles or phagosomes.
Our conclusion that CQ/Mon treatment damages early endosomes is in agreement with the report by Fraser et al. (2019 EMBO Rep) who also reported Gal8 to colocalize with EEA1-positive endosomes upon Mon treatment and observed loss of ATG16L1 colocalization with EEA1 in ATG13-deleted cells. Mauthe et al. (2018) found enhanced LDH sequestration upon CQ treatment indicating the involvement of (canonical) phagophores.

To demonstrate more clearly that CQ treatment damages early endosomes, we will include new results showing localization also of Gal8 and p62 to Rabaptin5-positive swollen endosomes and quantitation of Gal3 and Gal8 colocalization with Rabaptin5 (see below). As an additional marker for early endosomes, we assessed colocalization of Rabaptin5 with transferrin receptor (see below). We further determined degradation of transferrin receptor upon CQ treatment (see below).
We also have data showing that knockdown of Gal3 or p62 inhibits CQ-induced WIPI2 puncta (see below).

While the formation of WIPI2 puncta upon CQ treatment was strongly dependent on knockdown of FIP200, ATG13, or Rabaptin5, formation of LC3 puncta was less affected. We interpreted this relative insensitivity of LC3 puncta by the inhibition of autophagic flux by CQ/Mon that causes accumulation of LC3 structures produced by residual autophagy. As the reviewers point out, it might be due to LAP-like autophagy induced in parallel. However, complete ULK inhibition by MRT68921 (Fig. 4C and D) or FIP200 knockout (Fig. 6B and C) abolished CQ-induced LC3 structures, suggesting that – unlike on phagosomes or entotic vacuoles – there is little LAP-like autophagy. *This we will more clearly state in the text.*

Another distinction between canonical and LAP-like autophagy is BafA1 sensitivity: BafA1 added simultaneously with CQ inhibited LC3 recruitment to entotic vacuoles and phagosomes, i.e. LAP-like autophagy (Florey et al., 2015), while starvation induced LC3 activation was not inhibited. Yet, CQ-induced general LC3 lipidation was shown not to be blocked by the presence of BafA1, but rather enhanced (2 h incubation), and BafA1did not inhibit CQ-induced LDH sequestration (Mauthe et al., 2018), indicating that CQ (also) induces canonical BafA1-resistant autophagy. BafA1 added simultaneously with Mon did not reduce LC3 colocalization with EEA1-positive endosomes compared to Mon alone (Fraser et al., 2019 EMBO Rep).

We now performed additional experiments where BafA1 added together with chloroquine had no inhibitory effect on LC3B lipidation, supporting the view that chloroquine stimulates canonical autophagy (see below).

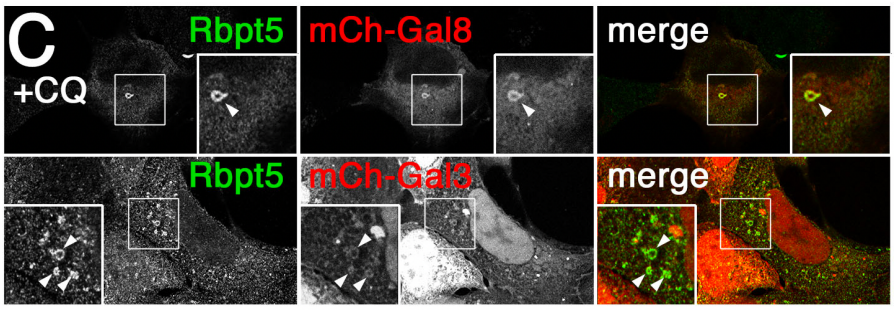
Point-by-point response to reviewer comments

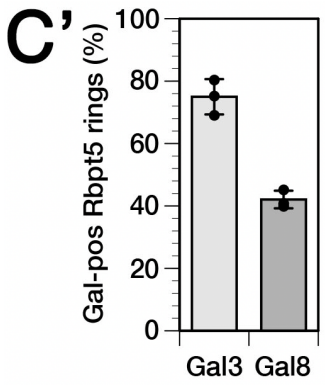
Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the current manuscript, Millarte et al reports a novel role of Rabaptin5 in selectively clearing damaged endosomes via canonical autophagy. They have identified FIP200 as a novel interactor of Rabaptin5 under basal conditions using yeast-two hybrid screening and further confirmed the interaction of Rabaptin5 with FIP200 with immunoprecipitation. They next used Chloroquine and monitored colocalization of the Rabaptin5 with WIPI2, ATG16L1 and LC3B to demonstrate the potential interaction of Rabaptin5 with the autophagic machinery. They have primarily used Gal-3 as a marker of membrane damage after 30 minutes of Chloroquine treatment. In order to further elucidate the role of Rabaptin5 in autophagic induction mediated by Chloroquine, they have silenced Rabaptin5, FIP200, ULK1 and ATG13 and observed a decrease in the number of LC3 or WIPI2 autophagosome formation. Based on these observations they tested if Rabaptin5 interacts with ATG16L1 upon Chloroquine treatment and confirmed their interaction with potential interaction sites of both Rabaptin5 with ATG16L1 with IP. The authors confirmed the interaction of Rabaptin5 with ATG16L1 by complementing the KO line with the mutant form of Rabaptin5 containing alanine residues in its consensus motif. Finally, they have used Salmonella and SCV as a model to study the role of Rabaptin5 in endomembrane damage and monitored a 50% decrease in the removal of Salmonella in Rabaptin5 KO or KD cells.

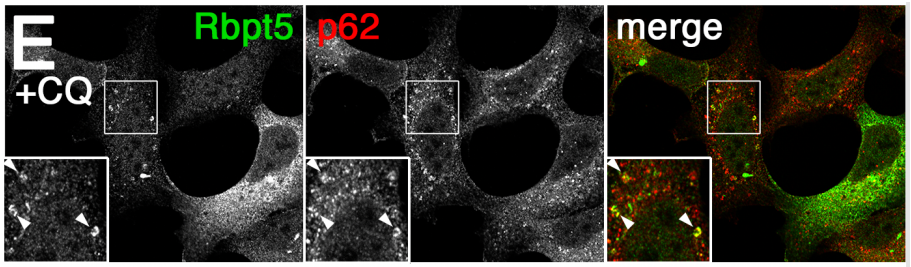
Major concerns
One of the major concerns is the membrane damage reported by chloroquine which is known to induce lysosomal swelling and further targeting of the swollen compartments to degradation by direct conjugation of LC3 onto single membrane as a form of non-canonical autophagy. The evidence regarding membrane damage by Gal3 colocalization on the Rabaptin5 vesicles is preliminary. As suggested by the authors the canonical autophagy pathway recognizing damaged membranes recruits also ALIX to the damaged membrane which was not observed in Supplementary Figure 2. The link to membrane damage by chloroquine and monensin with Rabaptin5 is not convincing as there is not sufficient evidence of membrane damage. In relation to this issue authors should consider using other damage markers as Gal8, p62 or NDP52 to provide additional claim with respect to membrane damage induced by chloroquine.

To expand on the question of CQ treatment damaging early endosomes, we also tested for Gal8 on Rabaptin5-positive enlarged endosomes and quantified the fraction of Rabaptin5-positive rings positive for Gal3 and Gal8 after 30 min of CQ treatment. We propose to include this data in Figure 2:

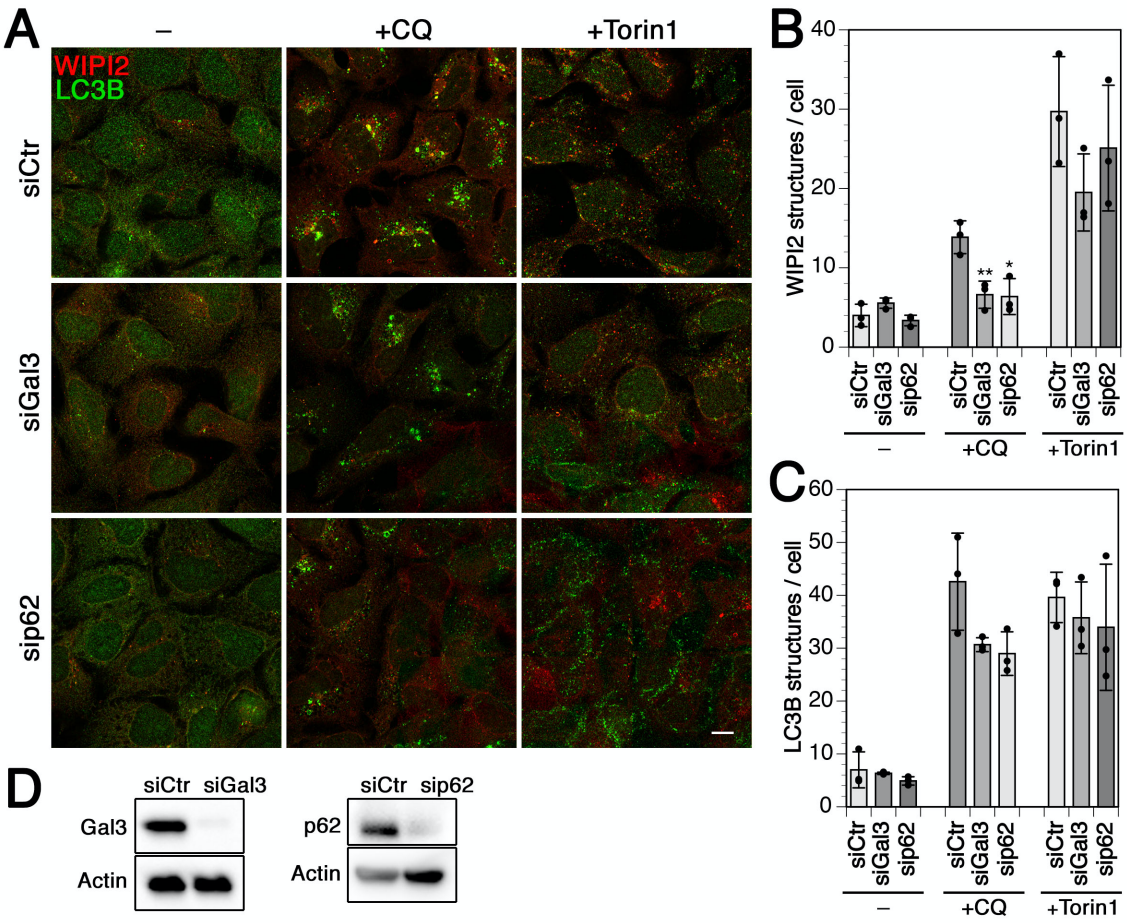




We furthermore colocalized p62 with Rabaptin5-positive enlarged endosomes after 30 min of CQ treatment. We propose to include this data in Figure 2:



We have also tested the importance of Gal3 and p62 by siRNA-mediated knockdown where we found a robust inhibition of induction of WIPI2 puncta with CQ, but not with Torin1. Formation of LC3 puncta was less reduced, similar to knockdowns of FIP200, ATG13, or Rabaptin5.
We propose to add these knockdown experiments as a supplementary figure:

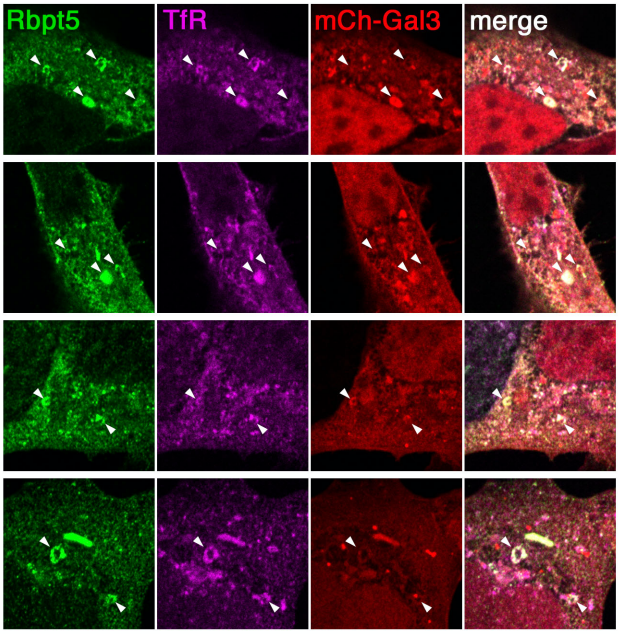


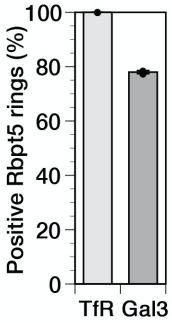
Chloroquine-induced autophagy involves galectin3 and p62.
A*: HEK293A cells were transfected with nontargeting siRNA (siCtr) or siRNAs silencing galectin3 (siGal3) or p62 (sip62) for 72 h and treated without (–) or with 60 µM chloroquine (+CQ) or 250 nM Torin1 for 150 min. Cells were fixed and immunostained for endogenous WIPI2 and LC3B. Bar, 10 µm.*
B and C*: WIPI2 (B) or LC3B puncta per cell (C) were quantified for each condition (mean and standard deviation of three independent experiments; two-tailed Student's t test: *p < 0.05, **p < 0.01, ***p<0.001).*
D*: Efficiency of galectin3 and p62 knockdown was assayed by immunoblotting.*

One of the main claims here is that Rabaptin5 regulates the targeting of damaged endosomes to autophagy.

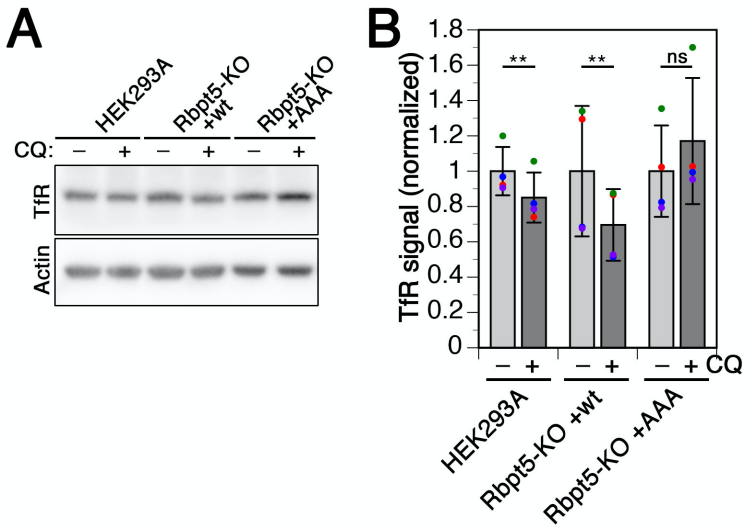
Clearly, these are early endosomes as stated in the abstract. However, the evidence presented here showing these are early endosomes is not convincing. Analysing Gal3 and Gal8 positive vesicles that are Rabaptin5 positive and an early endosomal marker will be important in this context. For example, there need to be additional evidence showing that early endosomes are targeted to autophagy. Is the degradation of TfR affected by this targeting? Did the authors look at the effect of Bafilomycin A1? If this process affects exclusively early endosomes, it should be BafA1 independent. This will direct more into the cellular function of this process.

Rabaptin5 is a bona fide marker of Rab5-positive early sorting endosomes. As a control, we confirmed colocalization of Rabaptin5 with transferrin receptor, another endosomal marker, on CQ-induced rings (Fig. 2B). We now also analyzed swollen endosomes with triple-staining for Rabaptin5, transferrin receptor, and Gal3 as shown in this gallery (30 min CQ, as in Fig. 2). All Rabaptin5-positive swollen endosomes (rings) were positive for transferrin receptor and ~80% for mCherry-Gal3.



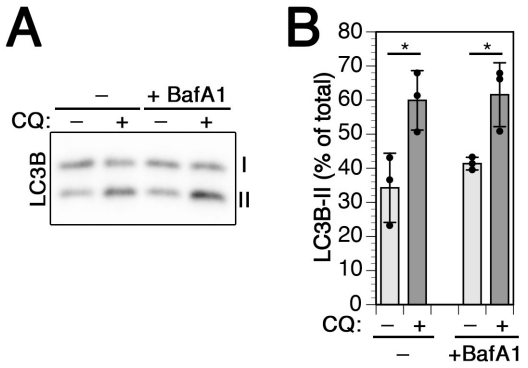


We further tested transferrin receptor levels with and without CQ. Since CQ inhibits autophagic flux, this assay may not be very sensitive. Nevertheless, we found a significant reduction of ~15% and ~30% after overnight incubation with CQ in parental HEK293A cells and in Rbpt5-KO cells re-expressing wild-type Rabaptin5, resp., but no reduction in Rbpt5-KO cells expressing the Rabaptin5-AAA mutant defective in binding to ATG16L1:



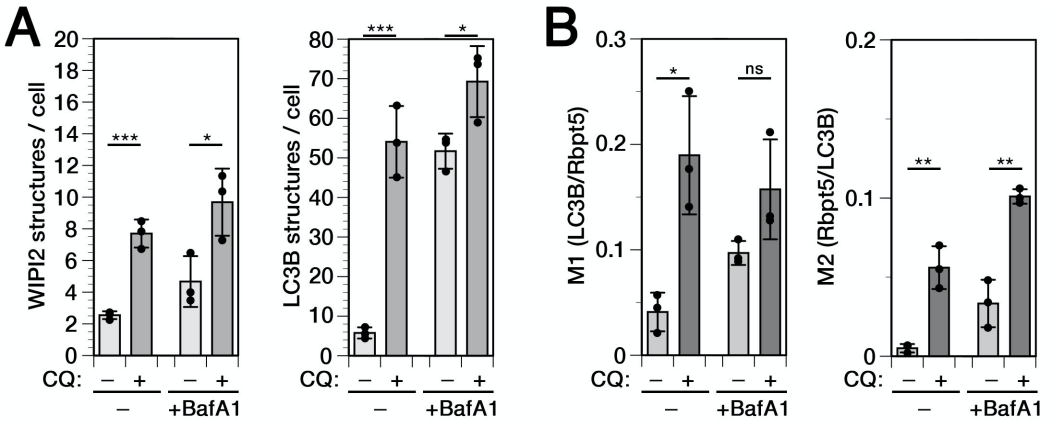
Transferrin receptor degradation upon chloroquine treatment.
A: *The indicated cell lines were incubated with or without 60 µM chloroquine (CQ) overnight before immunoblot analysis for transferrin receptor (TfR) and actin.*
B: *The TfR signal was quantified and normalized to the signal of actin. The mean and standard deviation of four independent experiments is plotted; two-tailed, paired Student's t test: ns not significant, **p < 0.01).*

As to the effect of BafA1, see our general response on top. Consistent with literature, we find increased LC3B lipidation already within 30 min of CQ treatment with and without BafA1:



Bafilomycin A1 does not inhibit chloroquine-induced LC3B lipidation.
*HEK293A cells were incubated without or with 60 µM chloroquine and 500 nM bafilomycin A1 for 30 minand non-lipidated and lipidated LC3B (I and II, resp.) were assayed by immunoblot analysis (A). The fraction of LC3B-II of total LC3B was quantified (mean and standard deviation of three independent experiments; two-tailed Student's t test: *p < 0.05).*

Upon longer incubations, LC3B lipidation is very strong already with BafA1 alone so that the effect of CQ cannot be assessed anymore, since both drugs inhibit autophagic flux.
Furthermore, we found a CQ-dependent increase in WIPI2- and LC3B-positive puncta to be insensitive to BafA1 (panel A below). Colocalization of Rabaptin5 to LC3B and LC3B to Rabaptin5 significantly increased upon CQ treatment independently of the presence of BafA1, indicating that at least a large part of CQ-induced LC3B puncta is not due to LAP-like autophagy.



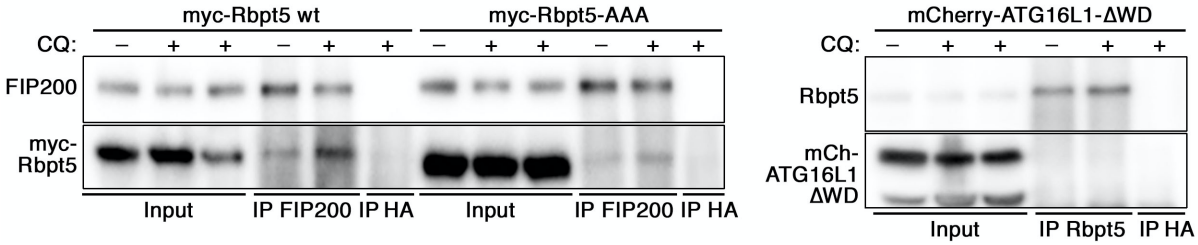
Effect of Bafilomycin A1 on chloroquine-induced WIPI2 and LC3B puncta and LC3B colocalization with Rabaptin5. *HEK293A cells were incubated with or without 60 μM chloroquine and/or 500 nM BafilomycinA1 for 150 min before immunofluorescence localization of WIPI2, LC3B, or Rabaptin5. In B, Manders' colocalization coefficients were determined, M1 showing the fraction of LC3B-positive structures also positive for Rabaptin5 and M2 showing the inverse The mean and standard deviation of three independent experiments is plotted; two-tailed Student's t test: ns not significant, *p < 0.05, **p < 0.01, ***p < 0.001).*

Minor concerns
Both for Figure 2 and Supplementary Figure 7 it will be clearer to have the images in colour rather than black and white for better interpretation.

We thought the grayscale images were clearer, but are happy to provide color images.

The interaction of FIP200 and ATG16L1 with Rabaptin5 is well characterized with immunoprecipitation and imaging but the interaction of Rabaptin5 in presence of chloroquine with FIP200 and ATG16L1 ΔWD are missing and it will be important to include if in the presence of chloroquine these interactions will increase or not.

We have now performed these co-IP experiments with and without CQ treatment. We find that Rabaptin5 wild-type and AAA mutant bind FIP200, somewhat stimulated by CQ treatment (left panel). ATG16L1-ΔWD did not co-immunoprecipitate with Rabaptin5, even when incubated with CQ (right panel). We are currently repeating these experiments for quantitation.



In order to further support the role of Rabaptin5 for LC3 lipidation upon chloroquine induced membrane damage, western blots of WT, +Rabaptin5, Rabaptin5 KO, Rabaption5 KO +WT or +AAA cell lines were analysed. However, the lysates were collected upon 30 minutes of chloroquine treatment which does not correlate with the imaging performed in Figure 2 as the number of LC3 vesicles did not show an increase upon 30 minutes of chloroquine treatment. The authors should include the 150 minutes time point for the LC3 lipidation in these conditions.

Because CQ inhibits autophagic flux, LC3-II accumulates after longer times in all cell lines. The differences can only be seen early.

The experiments with Salmonella are of great quality. The relationship of Rabaptin5 with SCV and the endomembrane damage induced by Salmonella could be further elucidated with Rabaptin5 positive vesicles at early infection stages. It is not very clear from the text how authors link the endosomal network previously described for chloroquine with infection. It would be important here to show that Salmonella mutants unable to damage endosomal membranes do not have an effect. In Figure 7 panel C, the time points on graphs are in hours but it should be in minutes. corrected.

Since Salmonella require T3SS for infection of HEK cells and T3SS causes the membrane damage, the proposed experiment is difficult.

The events of targeting the damaged membranes for degradation was well characterized by the recognition of these membranes by Gal3, Gal8 and recruitment of autophagic receptors to the site of damage (Chauhan et al. 2016; Jia et al. 2019; Thurston et al. 2012; Maejima et al. 2013; Kreibich et al. 2015). This manuscript introduces a new potential platform for the formation of autophagic machinery on endosomes with the interaction of Rabaptin5 with FIP200 and ATG16L1, however more evidence is required to link this to the clearance of damaged membranes. Previously it was shown that endolysosomal compartments that were neutralized and swollen by monensin and chloroquine had been directed to degradation by direct conjugation of LC3 to single membranes via noncanonical autophagy, but here authors propose another mechanism for this event via canonical autophagy.

As discussed in the general response above, the literature reports CQ and Mon to initiate both canonical autophagy and LAP-like autophagy, the latter particularly on phagosomes containing latex beads or entotic vacuoles. Our results – including the additional data above –concern the effects of CQ and Mon damaging early endosomes and causing recruitment of galectins and ubiquitination, triggering autophagy dependent on the ULK complex and WIPI2 as hallmarks of canonical autophagy, and Rabaptin5. The reviewer comments highlighted the possibility of LAP-like autophagy occurring in parallel, perhaps on endosomes that are not broken, which might explain the relative insensitivity of LC3 puncta induced by CQ and Mon – compared to the strong and robust reduction of WIPI2 puncta – on the knockdown of FIP200, ATG13, or Rabaptin5. In an alternative explanation, inhibition of autophagic flux causes remaining canonical autophagy to accumulate, while WIPI2 puncta are strongly inhibited. In support of the latter interpretation, ULK inhibition by MRT68921 (Fig. 4C and D) or FIP200 knockout (Fig. 6B and C) abolished CQ-induced LC3 structures, suggesting that – unlike on phagosomes or entotic vacuoles – there is little LAP-like autophagy. We propose to revise the manuscript to discuss these considerations more clearly.

Reviewer #1 (Significance (Required)):

Overall this work is very novel and shows some evidence of early endosomal autophagy. It could be relevant for some for of receptor-mediated signalling (although it is not discussed by the authors)
My experience is in intracellular trafficking of pathogens and membrane damage.

Referee Cross-commenting

In my opinion, the only way you can distinguish between double or single membrane is by EM. For me, the important part is to show this is targeting of early endosomes to autophagy, either using other early endosomal markers, analysing by WB some early endosome receptors such as TfR or other inhibitors. If the authors are able to address some these comments, I agree the paper will be in a better position for publication.

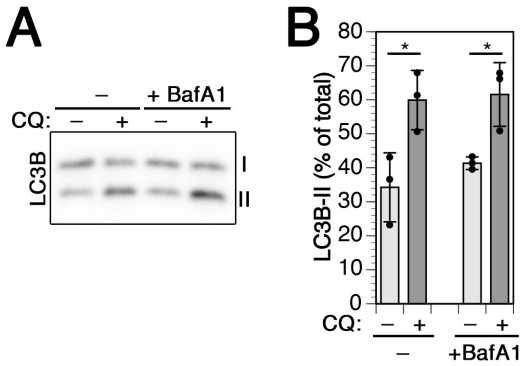
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Millarte et al study the role of Radaptin-5 (Rbpt5) during early endosome damage recognition by autophagy. The authors focus on using chloroquine (CQ) as an agent to induce endosomal swelling/damage and suggest that Rbpt5 is required for the recruitment of the autophagy machinery to perturbed endosomes. They further use salmonella infection as a model to confirm the role of Rbpt5 in this process. The authors initially show that Rbpt5 binds to FIP200 and subsequently focus on its interaction with ATG16L1 and identify a mutant that is unable to bind ATG16L1 or mediate the recognition of early endosomes by autophagy. Overall, this is an interesting study which provides molecular insights of how early endosomes can be targeted by the autophagy machinery where Rbpt5 may act as an autophagy receptor. Some specific comments are as follows:

Fig.3A: siRbpt5 seems to induce the localization of LC3 to ring-like structures during CQ treatment. Are these LAP-like structures (e.g. sensitive to BafA1 treatment)? And were they included in the quantification in Fig.3C?

Ring-like LC3 structures were also counted.
As discussed in the general remarks above, it is a possibility that knockdown-resistent LC3 recruitment (particularly rings) is due to a CQ-induced LAP-like process. The alternative explanation is that there is residual canonical autophagy upon knockdown of Rabaptin5, ATG13, or FIP200: while WIPI2 puncta are strongly reduced, LC3-positive structures accumulate due to inhibition of autophagic flux. In support of the latter interpretation, ULK inhibition by MRT68921 (Fig. 4C and D) or FIP200 knockout (Fig. 6B and C) abolished CQ-induced LC3 puncta or rings. We are currently doing experiments, as suggested, to test BafA1 sensitivity of CQ-induced LC3 ring-like structures in Rabaptin5 knockdown cells.

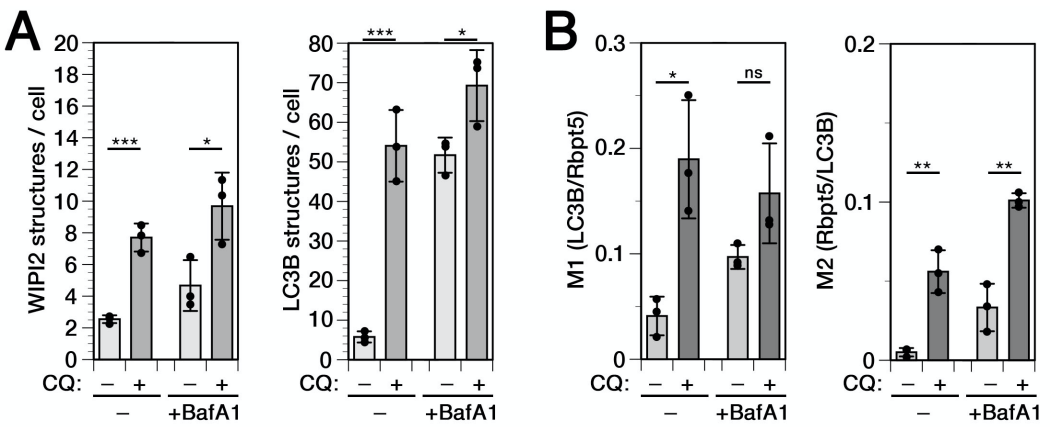
We now also analyzed BafA1 sensitivity of CQ-induced LC3B lipidation. Consistent with literature, we find increased LC3B lipidation already within 30 min of CQ treatment with and without BafA1.



Bafilomycin A1 does not inhibit chloroquine-induced LC3B lipidation.
*HEK293A cells were incubated without or with 60 μM chloroquine and 500 nM bafilomycin A1 for 30 minand non-lipidated and lipidated LC3B (I and II, resp.) were assayed by immunoblot analysis (A). The fraction of LC3B-II of total LC3B was quantified (mean and standard deviation of three independent experiments; two-tailed Student's t test: *p < 0.05).*

Upon longer incubations, LC3B lipidation is very strong already with BafA1 alone so that the effect of CQ cannot be assessed anymore, since both drugs inhibit autophagic flux.

Furthermore, we found a CQ-dependent increase in WIPI2- and LC3B-positive puncta to be insensitive to BafA1 (panel A below). Colocalization of Rabaptin5 to LC3B and LC3B to Rabaptin5 significantly increased upon CQ treatment independently of the presence of BafA1, indicating that at least a large part of CQ-induced LC3B puncta is not due to LAP-like autophagy.



Effect of Bafilomycin A1 on chloroquine-induced WIPI2 and LC3B puncta and LC3B colocalization with Rabaptin5. *HEK293A cells were incubated with or without 60 μM chloroquine and/or 500 nM BafilomycinA1 for 150 min before immunofluorescence localization of WIPI2, LC3B, or Rabaptin5. In B, Manders' colocalization coefficients were determined, M1 showing the fraction of LC3B-positive structures also positive for Rabaptin5 and M2 showing the inverse The mean and standard deviation of three independent experiments is plotted; two-tailed Student's t test: ns not significant, *p < 0.05, **p < 0.01, ***p < 0.001).*

Fig.4A&B: Since Rbpt5 KD has a weak effect on LC3 puncta formation (Fig.3) and to distinguish the effects of CQ in inducing LAP, the effects of ATG13 and ULK1 KD should be assessed by localising Rbpt5 with WIPI2 or ATG16L1.

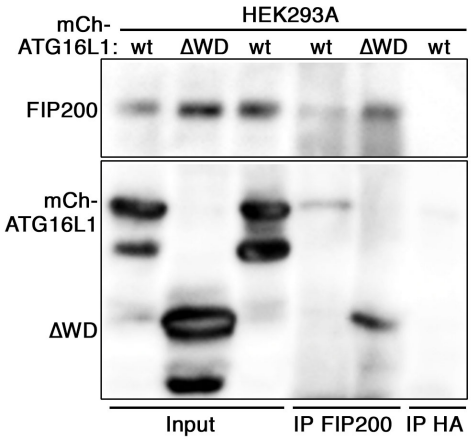
We are currently assessing that.

Fig.4: It is not clear why ULK1 KD would affect Torin1-induced autophagy but not LC3/WIPI2 localisation during CQ induced early endosome-damage. As the ULK inhibitors can target other pathways, the authors should confirm this finding in ULK1/2 double KO or KD cells.

We have used MRT68921, because it is frequently used in the literature for this purpose with high specificity. It was used for example by Lystad et al. (2019) together with VPS34IN1 to block all canonical autophagy to analyze exclusively noncanonical effects of monensin treatment. We could perform ULK1/2 double knockdowns, but since ULK2 cannot be detected on immunoblots in HEK293 cells, the result would be interpretable only when there is an effect.

Fig.5: The contribution of FIP200 in the interaction between Rbpt5 and ATG16L1 is unclear. Is binding between Rbpt5 and ATG16L1 mediated by ATG16L1's interaction with FIP200? The plasmid details describing the delta-WD40 deletion plasmid used in this study are missing and could be important to confirm that the detla-WD40 still retains binding to FIP200.

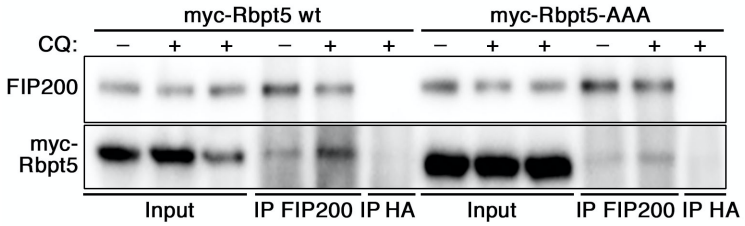
We will of course include the details on the deletion plasmid, which were missing by mistake. Our WD deletion construct of ATG16L1 consists of residues 1–319, precisely deleting only the WD40 repeats, but retaining the FIP200 interaction sequence and the second membrane binding segment (β).
We did a co-immunoprecipitation experiment and found both wild-type ATG16L1 and the ΔWD mutant to co-immunoprecipitate with FIP200:



We are currently repeating the experiment for quantitation.

Fig.5E: the authors should test Rbpt5 AAA mutant binding to FIP200. Since the mutant appears to express less, its binding to ATG16L1 should be quantified or repeated with more comparable expression levels.

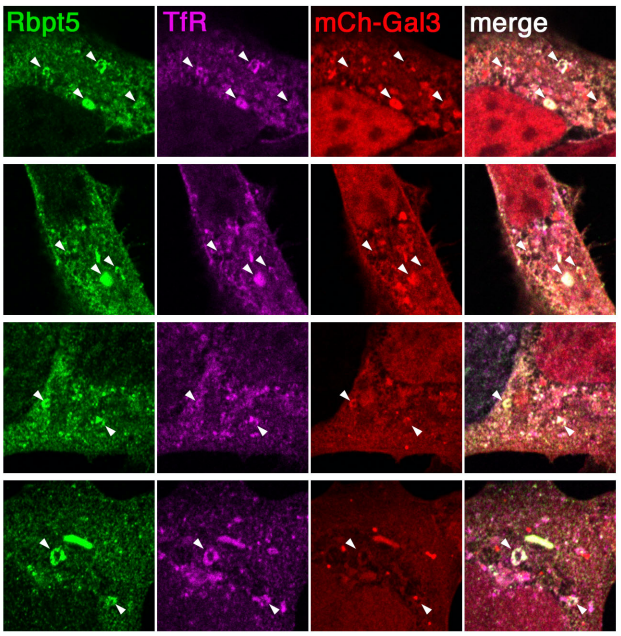
We have performed the suggested immunoblot showing that wild-type Rabaptin5 and the AAA mutant are both co-immunoprecipitated with FIP200. The interactions of both appear to be stimulated by CQ treatment. We are currently repeating the experiment for quantitation.

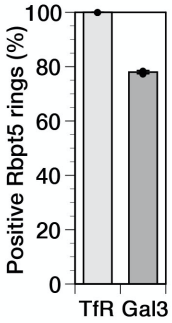


We have furthermore quantified the co-immunopreciptation experiments: Co-immunoprecipitation of ATG16L1ΔWD with Rabaptin5 was reduced to 6.6±2.1% and 3.4±2.1% in HEK293A and HeLa cells, respectively, relative to that of full-length ATG16L1 (signals normalized to that of the immunoprecipitated protein; mean and standard deviation of three independent experiments each).
Co-immunoprecipitation of ATG16L1 with Rabaptin5-AAA triple mutant was reduced to 1.5±1.2% relative to that with wild-type Rabaptin5 (signals normalized to that of the immunoprecipitated protein; mean and standard deviation of three independent experiments).

Fig.6: CQ treatment can induce various endosomal damage (in addition to early endosomes) and LC3 lipidation processes (e.g. LAP-like). The authors show that Rbpt5 is specifically involved in damaged early endosome autophagy. In this figure, it would be important to distinguish CQ-induced LC3 puncta as a result of early endosome damage or other lipidation processes (e.g. canonical or non-canonical autophagy). The use of FIP200 KO cells shows that LC3 puncta is inhibited. However, here a specific readout to look at early endosome recognition by autophagy is important. The authors can localize early endosome markers (EEA1) with autophagy players (e.g. WIPI2 and LC3). This is also relevant to other figures (e.g. supplementary figure 7E).

Rabaptin5 is a bona fide marker of Rab5-positive early sorting endosomes. As a control, we confirmed colocalization of Rabaptin5 with transferrin receptor, another endosomal marker, on CQ-induced rings (Fig. 2B). We also analyzed swollen endosomes with triple-staining for Rabaptin5/ transferrin receptor/ Gal3 as shown in this gallery (30 min CQ, as in Fig. 2). All Rabaptin5-positive swollen endosomes (rings) were positive for transferrin receptor and ~80% for mCherry-Gal3.





Our results are in agreement with Fraser et al. (2019) where they use EEA1 as an endosomal marker upon monensin treatment.

We also performed a colocalization analysis for Rabaptin5 and LC3B, showing enhanced colocalization after CQ treatment for 150 min: ~20% of LC3B is positive for Rabaptin5 upon CQ treatment:

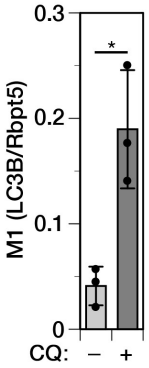


Fig.6F&G: the authors should show representative images of these localization images quantified here. These can be added in the supplementary figures.

We are happy to do this.

Minor comments:

Fig.2E: FIP200 seems to be highly overexpressed in this image. Commercial antibodies that recognise endogenous FIP200 are widely used and should be tested to confirm the colocalisation between FIP200 and Rbpt5.

We tested three anti-FIP200 antibodies, unfortunately none of them worked for immunofluorescence.

Fig.7C image: the different setting denoted by +/-, +/+ ..etc are not clearly defined.

We will improve this.

Reviewer #2 (Significance (Required)):

This is a interesting study and provides important mechanistic insights underlying the recognition of perturbed early endosomes by the autophagy machinery. Researchers interested in endosomal trafficking or autophagic substrate recognition are likely to benefit from this study.

Referee Cross-commenting

In my opinion, the authors have attempted to distinguish single membrane from double membrane LC3 lipidation by looking at the ULK complex requirement. As other reviewers suggested, this can be further confirmed by using ATG16L1 mutants. It is important however that these experiments are supplemented by co-localising autophagy proteins with alternative early endosome markers when Rbpt5 is inhibited.

As we point out to Reviewer #3, we did not refer to Lystad et al. (2019), because they analyzed different ATG16L1 mutants on their contribution to monensin-induced processes on LC3 lipidation after completely blocking canonical autophagy with the ULK inhibitor MRT68921 and/or the VPS34 inhibitor VPS34IN1. The Rabaptin5-dependent CQ-induced processes are blocked by MRT68921 (Fig. 4C).
Lystad et al. (2019) concludes that the WIPI binding sequence 207–230 is required for canonical autophagy, but not for LAP-like autophagy. We already show that knockdown of WIPI2 prevents localization of ATG16L1 to Rabaptin5-positive CQ-damaged endosomes (Suppl. Figure S4). Lystad et al. in addition show that deleting the β insert in ATG16L1 reduces non-canonical LC3 lipidation, while it does not affect canonical autophagy. If it is considered necessary, we could analyze localization of ATG16L1α with Rabaptin5 or transferrin receptor upon CQ treatment in wild-type and Rabaptin5-KO cells

I think if the authors are able to address the suggested experiments, this would help improve the manuscript and make it suitable for publication.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Millarte and colleagues find that Rabaptin5, a critical regulator of Rab5 activity, and a protein localized to early endosomes, interacts with FIP200 and ATG16L1. This interaction is confirmed and validated by a number of approaches (yeast 2 H, co-immunoprecipitation) and the binding sites of Rabaptin5 are mapped on FIP200 and ATG16L1. More precisely the binding site for ATG16L1 is nicely mapped on Rabaptin 5 by analogy with other ATG16L1 binders. The authors investigate the significance of this binding of Rabaptin5 to the autophagy proteins by proposing this interaction is required for targeting "autophagy to damaged endosomes". Endosomes are damaged with short treatments of chloroquine, a well studied compound previously shown to inhibit autophagy by disrupting fusion of autophagosomes with lysosomes. They propose the recruitment of autophagy (proteins) to the damaged endosomes may allow them to be eliminated. They use another model (phagocytosis of salmonella) to probe the role for rabaptin5 and its partners FIP200 and ATG16L1 in the well-documented role of autophagy on the elimination of salmonella in SCVs (Salmonella containing vacuole) formed from endosomes. Using short infection time points, and the Rabaptin5 mutants which prevent ATG16L1 binding they suggest Rabaptin5 binding contributes to elimination and killing of Salmonella by recruitment of ATG16L1.

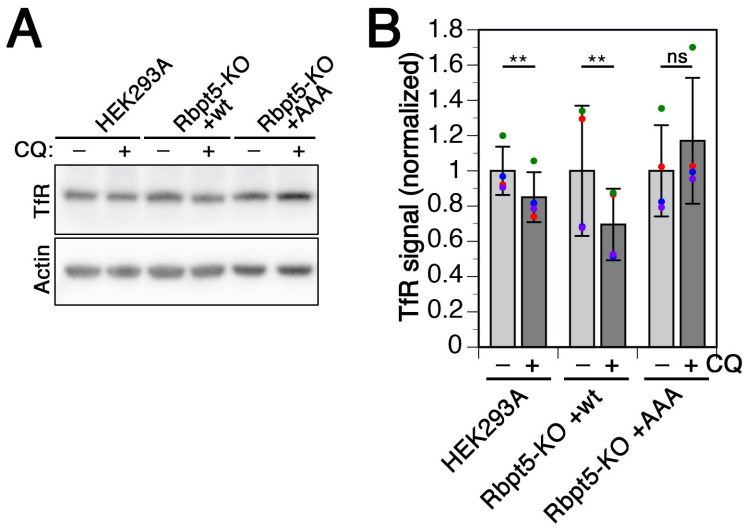
Major comments:

1. The authors make an unfortunate and confusing choice of wording in the title and the text of "autophagy being recruited" to damaged early endosomes. A protein can recruit another protein but it can not recruit a process or pathway to a membrane.

In the title we use the term "target". It is OK for us to avoid the expression "recruiting autophagy".

2. The authors conclude that Rabaptin5 may have a role in autophagy directed to damaged early endosomes. The conclusion that Rabaptin5 binds FIP200 and ATG16L1 are convincing. The main issue is however in identifying what sort of process they are following. They have shown WIPI2 and LC3 can be recruited to early endosomes after 30 min chloroquine treatment but there is no data to explain the consequences of the binding of these proteins. They do not provide proof that canonical autophagosomes are formed which engulf and remove the damaged endosomes, nor do they show that the recruitment of WIPI2 is to a single membrane (presumably damaged early endosomes) which would be a non-canonical pathway. They often use the terminology "chloroquine-induced autophagy" (see Figure 4) but have virtually no proof they have induced either canonical or non-canonical pathways in their experiments. The only evidence they provide that there is some alteration in a membrane-mediated event is increase in lipidation of LC3 in Figure 6.
The authors must follow either an early endosome protein or cargo to demonstrate lysosome-mediated degradation indicative of autophagy, or demonstrate the process is a variation on non-canonical autophagy.

We analyzed transferrin receptor levels with and without CQ to test degradation of an early endosomal marker protein. Since CQ inhibits autophagic flux, this assay may not be very sensitive. Nevertheless, we found a significant reduction of ~15% and ~30% after overnight incubation with CQ in parental HEK293 cells and in Rbpt5-KO cells re-expressing wild-type Rabaptin5, resp., but no reduction in Rbpt5-KO cells expressing the Rabaptin5-AAA mutant defective in binding to ATG16L1:



Transferrin receptor degradation upon chloroquine treatment.
A: *The indicated cell lines were incubated with or without 60 µM chloroquine (CQ) overnight before immunoblot analysis for transferrin receptor (TfR) and actin.*
B: *The TfR signal was quantified and normalized to the signal of actin. The mean and standard deviation of four independent experiments is plotted; two-tailed, paired Student's t test: ns not significant, **p < 0.01).*

2. There are concerns about the replicates done for many experiments in particular the co-immunoprecipitations which are not quantified (Figure 1 and 5).

We will provide the quantitation of these blots:
Fig. 1E–G:Co-immunoprecipitation of Rabaptin5, ATG13, and ULK1 with FIP200Δ280–440 was reduced to 14.0±9.5%, 47.8±12.9%, and 73.6±3.8%, respectively, relative to that with full-length FIP200 (signals normalized to that of the immunoprecipitated protein; mean and standard deviation of three independent experiments each).

Fig. 5B:

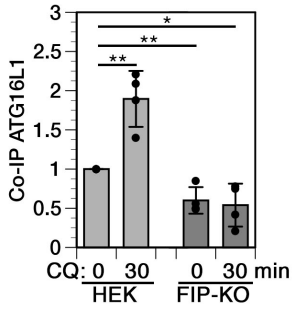


Fig. 5C: Co-immunoprecipitation of ATG16L1ΔWD with Rabaptin5 was reduced to 6.6±2.1% and 3.4±2.1% in HEK293A and HeLa cells, respectively, relative to that of full-length ATG16L1 (signals normalized to that of the immunoprecipitated protein; mean and standard deviation of three independent experiments each).

Fig. 5E: Co-immunoprecipitation of ATG16L1 with Rabaptin5-AAA triple mutant was reduced to 1.5±1.2% relative to that with wild-type Rabaptin5 (signals normalized to that of the immunoprecipitated protein; mean and standard deviation of three independent experiments).

3. The rescue experiments, even if done with stable cells lines made in the parental HEK293 cell line should be viewed with caution because of the very different amounts of Rabaptin5 (see Figure 6A). The overexpression of Rabaptin5 has not been well studied and comparisons with the mutants are therefore preliminary (Figure 6F and G).

Fig 6A shows that Rabaptin5 levels are similar except for +Rbpt, where they are higher, and R-KO, which has none. Additional Rabaptin5 seems not to significantly enhance early WIPI and ATG16L1 colocalization.

4. Conclusions about the role of the ULK complex, or ULK1 versus ULK2, should be expanded by studying the activity of the complex (phosphorylation of ATG13 for example) in order to make the conclusions more significant.

We consider this to be beyond the scope of this study. Rabaptin5-dependent autophagy depends on the components of the ULK complex.

Minor comments:

1. Much of the labelling in the immunofluorescence images is not visible even on the screen version.

We were careful to have the signals within the dynamic range of the image, but we can enhance the signals for better visibility.

2. The LC3-lipidation experiment (Figure 6D) should be re-analysed by normalization to the loading control. The result may be significantly different and is open to re-interpretation. The quality of this western blot is also very poor.

Quantitation was based on the ratio between LC3B-I and -II or the percentage of II of the total, always within the same lane and therefore largely independent of loading.

Reviewer #3 (Significance (Required)):

This manuscript topic fits into the field of study of canonical versus non-canonical autophagy. This literature is best described as "LAP" first discovered by Doug Green, (Sanjuan in 2009) but more recently as a phenomena induced by monesin, and viral infection amongst others. Most relevant to this study are the references (in the text) by Florey (Autophagy 2015), Fletcher (EMBO J, 2018) and others. However, this manuscript fails to cite and consider the critical findings in a key study published by Lystad et al., Nature Cell Biology 2019, which examines the role of ATG16 in both canonical and non-canonical autophagy. The current study if placed into the context of the Lystad study would have significantly more value, and potentially make the findings more significant.

We did not refer to Lystad et al. (2019), because they analyzed different ATG16L1 mutants on their contribution to monensin-induced processes on LC3 lipidation after completely blocking canonical autophagy with the ULK inhibitor MRT68921 and/or the VPS34 inhibitor VPS34IN1. The Rabaptin5-dependent CQ-induced processes are blocked by MRT68921 (Fig. 4C).
Lystad et al. (2019) concludes that the WIPI binding sequence 207–230 is required for canonical autophagy, but not for LAP-like autophagy. We already show that knockdown of WIPI2 prevents localization of ATG16L1 to Rabaptin5-positive CQ-damaged endosomes endosomes (Suppl. Figure S4). Lystad et al. in addition show that deleting the β insert in ATG16L1 reduces non-canonical LC3 lipidation, while it does not affect canonical autophagy. If it is considered necessary, we could analyze localization of ATG16L1α with Rabaptin5 upon CQ treatment.

Furthermore, the short chloroquine treatments used here could be of interest to the field if using the cited study of Mauthe et al., (which very clearly defines the effect of chloroquine after long (5 hrs treatment)) the authors would to revisit and repeat some of the key experiments in order to demonstrate the effects of 30 minute treatment. Does such short treatment block fusion? Does it affect the pH of the acidic compartments? Does it inactivate the endocytitic pathway? As the manuscript stands the lack of this understanding of the effect of chloroquine at short times, makes the observations difficult to be place into any biological context.

This reviewer has expertise in autophagy, autophagosome formation and is familiar with the areas of endocytosis and infection.

Referee Cross-commenting

I think a major concern about the manuscript which is present in all reviews is the lack of clarity about what type of membrane LC3 is added to- is this the damaged endosome or a forming autophagosome? This leads to the question of what type of process is being observed here? non-canonical versus canonical autophagy.

Point-by-point response to reviewer comments

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the current manuscript, Millarte et al reports a novel role of Rabaptin5 in selectively clearing damaged endosomes via canonical autophagy. They have identified FIP200 as a novel interactor of Rabaptin5 under basal conditions using yeast-two hybrid screening and further confirmed the interaction of Rabaptin5 with FIP200 with immunoprecipitation. They next used Chloroquine and monitored colocalization of the Rabaptin5 with WIPI2, ATG16L1 and LC3B to demonstrate the potential interaction of Rabaptin5 with the autophagic machinery. They have primarily used Gal-3 as a marker of membrane damage after 30 minutes of Chloroquine treatment. In order to further elucidate the role of Rabaptin5 in autophagic induction mediated by Chloroquine, they have silenced Rabaptin5, FIP200, ULK1 and ATG13 and observed a decrease in the number of LC3 or WIPI2 autophagosome formation. Based on these observations they tested if Rabaptin5 interacts with ATG16L1 upon Chloroquine treatment and confirmed their interaction with potential interaction sites of both Rabaptin5 with ATG16L1 with IP. The authors confirmed the interaction of Rabaptin5 with ATG16L1 by complementing the KO line with the mutant form of Rabaptin5 containing alanine residues in its consensus motif. Finally, they have used Salmonella and SCV as a model to study the role of Rabaptin5 in endomembrane damage and monitored a 50% decrease in the removal of Salmonella in Rabaptin5 KO or KD cells.

Major concerns
One of the major concerns is the membrane damage reported by chloroquine which is known to induce lysosomal swelling and further targeting of the swollen compartments to degradation by direct conjugation of LC3 onto single membrane as a form of non-canonical autophagy. The evidence regarding membrane damage by Gal3 colocalization on the Rabaptin5 vesicles is preliminary. As suggested by the authors the canonical autophagy pathway recognizing damaged membranes recruits also ALIX to the damaged membrane which was not observed in Supplementary Figure 2. The link to membrane damage by chloroquine and monensin with Rabaptin5 is not convincing as there is not sufficient evidence of membrane damage. In relation to this issue authors should consider using other damage markers as Gal8, p62 or NDP52 to provide additional claim with respect to membrane damage induced by chloroquine.

To expand on the question of CQ treatment damaging early endosomes, we performed the experiments suggested by the reviewer and also tested for the recruitment of Gal8 and p62 on Rabaptin5-positive enlarged endosomes and quantified the fraction of Rabaptin5-positive rings positive for Gal3 and Gal8 after 30 min of CQ treatment. The results show recruitment of both Gal8 and p62 to Rabaptin5-positive rings (added to Figure 2, as panels C and E, resp.). Quantitation (included Figure 2 as panel C') shows ~75% of swollen early endosomes to be positive for Gal3 and ~40% for Gal8.

We have also tested the importance of Gal3 and p62 by siRNA-mediated knockdown where we found a robust inhibition of induction of WIPI2 puncta with CQ, but not with Torin1. Formation of LC3 puncta was less reduced, similar to knockdowns of FIP200, ATG13, or Rabaptin5. This data was added as Appendix Figure S3.

These additional data strengthen the conclusion that CQ damages early endosomes and that galectins and p62 recruited to the sites of damage are important for subsequent autophagy.

One of the main claims here is that Rabaptin5 regulates the targeting of damaged endosomes to autophagy. Clearly, these are early endosomes as stated in the abstract. However, the evidence presented here showing these are early endosomes is not convincing. Analysing Gal3 and Gal8 positive vesicles that are Rabaptin5 positive and an early endosomal marker will be important in this context. For example, there need to be additional evidence showing that early endosomes are targeted to autophagy. Is the degradation of TfR affected by this targeting? Did the authors look at the effect of Bafilomycin A1? If this process affects exclusively early endosomes, it should be BafA1 independent. This will direct more into the cellular function of this process.

Rabaptin5 is a bona fide marker of Rab5-positive early sorting endosomes. Following the reviewer's suggestion, we further analyzed swollen endosomes by triple-staining for Rabaptin5, the early endosome-specific transferrin receptor, and Gal3 after 30 min of CQ treatment: all Rabaptin5-positive swollen

endosomes were positive for transferrin receptor and ~80% for mCherry-Gal3. This result is included as Figure EV2A and B.

We further tested transferrin receptor levels with and without CQ. Since CQ inhibits autophagic flux, this assay may not be very sensitive. Nevertheless, we found a significant reduction of ~15% and ~30% after overnight incubation with CQ in parental HEK293A cells and in Rbpt5-KO cells re-expressing wild-type Rabaptin5, resp., but no reduction in Rbpt5-KO cells expressing the Rabaptin5-AAA mutant defective in binding to ATG16L1:
These results were included as Appendix Figure S5D and E of the revised manuscript.

We tested the effect of BafA1 on CQ-induced LC3B lipidation: we found increased LC3B lipidation already after 30 min of CQ treatment both with and without BafA1 (in agreement with Mauthe et al, 2018). This result was included as Appendix Figure S3C. Upon longer incubations, LC3B lipidation is very strong already with BafA1 alone so that the effect of CQ cannot be assessed anymore.
Furthermore, we found the CQ-induced increase in WIPI2- and LC3B-positive puncta to be insensitive to BafA1 and colocalization of Rabaptin5 to LC3B and LC3B to Rabaptin5 to be significantly increased upon CQ treatment independently of the presence of BafA1, indicating that at least a large part of CQ-induced LC3B puncta is not due to LAP-like autophagy. These data were included in Appendix Figure S3 as panels D and E.

Minor concerns
Both for Figure 2 and Supplementary Figure 7 it will be clearer to have the images in colour rather than black and white for better interpretation.

We thought the grayscale images were clearer, but are happy to provide color images if requested.

The interaction of FIP200 and ATG16L1 with Rabaptin5 is well characterized with immunoprecipitation and imaging but the interaction of Rabaptin5 in presence of chloroquine with FIP200 and ATG16L1 ΔWD are missing and it will be important to include if in the presence of chloroquine these interactions will increase or not.

We have now performed these co-IP experiments with and without CQ treatment. We find that Rabaptin5 wild-type and AAA mutant bind FIP200, somewhat stimulated by CQ treatment (Figure 5, new panel H). ATG16L1-ΔWD did not co-immunoprecipitate with Rabaptin5, even when incubated with CQ (Figure 5, new panel D).

In order to further support the role of Rabaptin5 for LC3 lipidation upon chloroquine induced membrane damage, western blots of WT, +Rabaptin5, Rabaptin5 KO, Rabaption5 KO +WT or +AAA cell lines were analysed. However, the lysates were collected upon 30 minutes of chloroquine treatment which does not correlate with the imaging performed in Figure 2 as the number of LC3 vesicles did not show an increase upon 30 minutes of chloroquine treatment. The authors should include the 150 minutes time point for the LC3 lipidation in these conditions.

Because CQ inhibits autophagic flux, LC3-II accumulates after longer times in all cell lines. The differences can only be seen at early timepoints.

The experiments with Salmonella are of great quality. The relationship of Rabaptin5 with SCV and the endomembrane damage induced by Salmonella could be further elucidated with Rabaptin5 positive vesicles at early infection stages. It is not very clear from the text how authors link the endosomal network previously described for chloroquine with infection. It would be important here to show that Salmonella mutants unable to damage endosomal membranes do not have an effect. In Figure 7 panel C, the time points on graphs are in hours but it should be in minutes. corrected.

Since Salmonella require T3SS for infection of HEK cells and T3SS causes the membrane damage, the proposed experiment is very difficult.

The events of targeting the damaged membranes for degradation was well characterized by the recognition of these membranes by Gal3, Gal8 and recruitment of autophagic receptors to the site of damage (Chauhan et al. 2016; Jia et al. 2019; Thurston et al. 2012; Maejima et al. 2013; Kreibich et al. 2015). This manuscript introduces a new potential platform for the formation of autophagic machinery on endosomes with the interaction of Rabaptin5 with FIP200 and ATG16L1, however more evidence is required to link this to the clearance of damaged membranes. Previously it was shown that endolysosomal compartments that were neutralized and swollen by monensin and chloroquine had been directed to degradation by direct conjugation of LC3 to single membranes via noncanonical autophagy, but here authors propose another mechanism for this event via canonical autophagy.

The literature reports CQ and Mon to initiate both canonical autophagy and LAP-like autophagy, the latter particularly on phagosomes containing latex beads or entotic vacuoles. Our results – including the additional data above –concern the effects of CQ and Mon damaging early endosomes and causing recruitment of galectins and ubiquitination, triggering autophagy dependent on the ULK complex and WIPI2 as hallmarks of canonical autophagy, and Rabaptin5. The reviewer's comments highlights the possibility of LAP-like autophagy occurring in parallel, perhaps on endosomes that are not broken, which might explain the relative insensitivity of LC3 puncta induced by CQ and Mon – compared to the strong and robust reduction of WIPI2 puncta – upon knockdown of FIP200, ATG13, or Rabaptin5. In an alternative explanation, inhibition of autophagic flux causes remaining canonical autophagy to accumulate, while WIPI2 puncta are strongly inhibited. In support of the latter interpretation, ULK inhibition by MRT68921 (Figure 4E and F) or FIP200 knockout (Figure 6B and C) abolished CQ-induced LC3 structures, suggesting that – unlike on phagosomes or entotic vacuoles – there is little LAP-like autophagy. We discuss these considerations more clearly in the revised manuscript (Discussion).

Reviewer #1 (Significance (Required)):

Overall this work is very novel and shows some evidence of early endosomal autophagy. It could be relevant for some for of receptor-mediated signalling (although it is not discussed by the authors)
My experience is in intracellular trafficking of pathogens and membrane damage.

Referee Cross-commenting

In my opinion, the only way you can distinguish between double or single membrane is by EM. For me, the important part is to show this is targeting of early endosomes to autophagy, either using other early endosomal markers, analysing by WB some early endosome receptors such as TfR or other inhibitors. If the authors are able to address some these comments, I agree the paper will be in a better position for publication.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Millarte et al study the role of Radaptin-5 (Rbpt5) during early endosome damage recognition by autophagy. The authors focus on using chloroquine (CQ) as an agent to induce endosomal swelling/damage and suggest that Rbpt5 is required for the recruitment of the autophagy machinery to perturbed endosomes. They further use salmonella infection as a model to confirm the role of Rbpt5 in this process. The authors initially show that Rbpt5 binds to FIP200 and subsequently focus on its interaction with ATG16L1 and identify a mutant that is unable to bind ATG16L1 or mediate the recognition of early endosomes by autophagy. Overall, this is an interesting study which provides molecular insights of how early endosomes can be targeted by the autophagy machinery where Rbpt5 may act as an autophagy receptor. Some specific comments are as follows:

Fig.3A: siRbpt5 seems to induce the localization of LC3 to ring-like structures during CQ treatment. Are these LAP-like structures (e.g. sensitive to BafA1 treatment)? And were they included in the quantification in Fig.3C?

Ring-like LC3 structures were also counted.
It is a possibility that knockdown-resistent LC3 recruitment (particularly rings) is due to a CQ-induced LAP-like process. The alternative explanation is that there is residual canonical autophagy upon knockdown of Rabaptin5, ATG13, or FIP200: while WIPI2 puncta are strongly reduced, LC3-positive structures accumulate due to inhibition of autophagic flux. In support of the latter interpretation, ULK inhibition by MRT68921 (Figure 4E and F) or FIP200 knockout (Figure 6B and C) abolished CQ-induced LC3 puncta or rings.

In any case, we now tested the effect of BafA1 on CQ-induced LC3B lipidation and on WIPI2 and LC3B puncta. We found increased LC3B lipidation already after 30 min of CQ treatment both with and without BafA1 (in agreement with Mauthe et al, 2018). This result was included as Appendix Figure S3C. Upon longer incubations, LC3B lipidation is very strong already with BafA1 alone so that the effect of CQ cannot be assessed anymore.

Furthermore, we found the CQ-induced increase in WIPI2- and LC3B-positive puncta to be insensitive to BafA1 and colocalization of Rabaptin5 to LC3B and LC3B to Rabaptin5 to be significantly increased upon CQ treatment independently of the presence of BafA1, indicating that at least a large part of CQ-induced LC3B puncta is not due to LAP-like autophagy. These data were included in Appendix Figure S3 as panels D and E.
The BafA1 insensitivity argues against LAP-like processes.

Fig.4A&B: Since Rbpt5 KD has a weak effect on LC3 puncta formation (Fig.3) and to distinguish the effects of CQ in inducing LAP, the effects of ATG13 and ULK1 KD should be assessed by localising Rbpt5 with WIPI2 or ATG16L1.

Following the reviewer's suggestion, we analyzed the recruitment of ATG16L1 to enlarged Rabaptin5-positive endosomes: knockdown of ATG13 or inhibition of both ULK1 and ULK2 by MRT68921prevented ATG16L1 recruitment and blocked overall the colocalization of ATG16L1 and Rabaptin5. This result is presented in Appendix Figure S2.

Fig.4: It is not clear why ULK1 KD would affect Torin1-induced autophagy but not LC3/WIPI2 localisation during CQ induced early endosome-damage. As the ULK inhibitors can target other pathways, the authors should confirm this finding in ULK1/2 double KO or KD cells.

We have used MRT68921, because it is frequently used in the literature for this purpose with high specificity. It was used for example by Lystad et al. (2019) together with VPS34IN1 to block all canonical autophagy to analyze exclusively noncanonical effects of monensin treatment.
Following the reviewer's suggestion, we also performed ULK2- and ULK1/2 double knockdowns. As now shown in Figure 4C and D, ULK2 knockdown and even more so the ULK1/2 double knockdown reduced CQ-induced WIPI2 and LC3 puncta, indicating that CQ induction of autophagy depends to a large extent on ULK2.

Fig.5: The contribution of FIP200 in the interaction between Rbpt5 and ATG16L1 is unclear. Is binding

between Rbpt5 and ATG16L1 mediated by ATG16L1's interaction with FIP200? The plasmid details describing the delta-WD40 deletion plasmid used in this study are missing and could be important to confirm that the detla-WD40 still retains binding to FIP200.

The details of the deletion plasmid were missing by mistake and have been added now: the WD deletion construct of ATG16L1 consists of residues 1–319, precisely deleting only the WD40 repeats, but retaining the FIP200 interaction sequence and the second membrane binding segment (β).
We did co-immunoprecipitation experiments and found both wild-type ATG16L1 and the ΔWD mutant to co-immunoprecipitate with FIP200. Immunoblot and quantitation were added as Figure 5E and E'.

Fig.5E: the authors should test Rbpt5 AAA mutant binding to FIP200. Since the mutant appears to express less, its binding to ATG16L1 should be quantified or repeated with more comparable expression levels.

We have performed the suggested immunoblots showing that wild-type Rabaptin5 and the AAA mutant are both co-immunoprecipitated with FIP200, now shown in Figure 5H. We have furthermore quantified the co-immunopreciptation of ATG16L1 with Rabaptin5 wild-type or AAA mutant and added the result to the figure legend: 1.5±1.2% co-immunoprecipitation with AAA relative to wild-type Rabaptin5. Other co-immunoprecipitations were also quantified and included.

Fig.6: CQ treatment can induce various endosomal damage (in addition to early endosomes) and LC3 lipidation processes (e.g. LAP-like). The authors show that Rbpt5 is specifically involved in damaged early endosome autophagy. In this figure, it would be important to distinguish CQ-induced LC3 puncta as a result of early endosome damage or other lipidation processes (e.g. canonical or non-canonical autophagy). The use of FIP200 KO cells shows that LC3 puncta is inhibited. However, here a specific readout to look at early endosome recognition by autophagy is important. The authors can localize early endosome markers (EEA1) with autophagy players (e.g. WIPI2 and LC3). This is also relevant to other figures (e.g. supplementary figure 7E).

Rabaptin5 is a bona fide marker of Rab5-positive early sorting endosomes. As a control, we confirmed colocalization of Rabaptin5 with transferrin receptor, another endosomal marker, on CQ-induced rings (Figure 2A'). We also analyzed swollen endosomes with triple-staining for Rabaptin5/ transferrin receptor/ Gal3 after 30 min of CQ treatment: all Rabaptin5-positive swollen endosomes were positive for transferrin receptor and ~80% for mCherry-Gal3. This result is included as Figure EV2A and B.
Similalry, we used transferrin receptor to analyze endosomal recruitment of ATG16L1α and colocalization (Figure EV4C and D).

Fig.6F&G: the authors should show representative images of these localization images quantified here. These can be added in the supplementary figures.

We added such images in Figure EV4A.

Minor comments:

Fig.2E: FIP200 seems to be highly overexpressed in this image. Commercial antibodies that recognise endogenous FIP200 are widely used and should be tested to confirm the colocalisation between FIP200 and Rbpt5.

We tested three anti-FIP200 antibodies, unfortunately none of them worked for immunofluorescence.

Fig.7C image: the different setting denoted by +/-, +/+ ..etc are not clearly defined.

We have improved this by spelling out there meaning in the figure (e.g. TfR–/LC3B+).

Reviewer #2 (Significance (Required)):

This is a interesting study and provides important mechanistic insights underlying the recognition of perturbed early endosomes by the autophagy machinery. Researchers interested in endosomal trafficking or autophagic substrate recognition are likely to benefit from this study.

Referee Cross-commenting

In my opinion, the authors have attempted to distinguish single membrane from double membrane LC3 lipidation by looking at the ULK complex requirement. As other reviewers suggested, this can be further confirmed by using ATG16L1 mutants. It is important however that these experiments are supplemented by co-localising autophagy proteins with alternative early endosome markers when Rbpt5 is inhibited.

Lystad et al (2019) provided evidence that the WIPI binding sequence 207–230 in ATG16L1 is required for canonical autophagy, but not for LAP-like autophagy, and that deleting the β insert in full-length ATG16L1 reduces non-canonical LC3 lipidation, while it does not affect canonical autophagy.
We already show that knockdown of WIPI2 prevents localization of ATG16L1 to Rabaptin5-positive CQ-damaged endosomes (Figure EV3C and D). Following the reviewer's suggestion, we have now also analyzed the ability of ATG16L1α to be recruited to CQ-induced enlarged endosomes and to colocalize with transferrin receptor in wild-type and Rabaptin5-AAA mutant cells. We found that ATG16L1α is recruited as efficiently as the β isoform with wild-type, but not with the Rabaptin5 mutant defective in binding to ATG16L1. These results support canonical autophagy and are included in Figure EV4B–D.

I think if the authors are able to address the suggested experiments, this would help improve the manuscript and make it suitable for publication.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Millarte and colleagues find that Rabaptin5, a critical regulator of Rab5 activity, and a protein localized to early endosomes, interacts with FIP200 and ATG16L1. This interaction is confirmed and validated by a number of approaches (yeast 2 H, co-immunoprecipitation) and the binding sites of Rabaptin5 are mapped on FIP200 and ATG16L1. More precisely the binding site for ATG16L1 is nicely mapped on Rabaptin 5 by analogy with other ATG16L1 binders. The authors investigate the significance of this binding of Rabaptin5 to the autophagy proteins by proposing this interaction is required for targeting "autophagy to damaged endosomes". Endosomes are damaged with short treatments of chloroquine, a well studied compound previously shown to inhibit autophagy by disrupting fusion of autophagosomes with lysosomes. They propose the recruitment of autophagy (proteins) to the damaged endosomes may allow them to be eliminated. They use another model (phagocytosis of salmonella) to probe the role for rabaptin5 and its partners FIP200 and ATG16L1 in the well-documented role of autophagy on the elimination of salmonella in SCVs (Salmonella containing vacuole) formed from endosomes. Using short infection time points, and the Rabaptin5 mutants which prevent ATG16L1 binding they suggest Rabaptin5 binding contributes to elimination and killing of Salmonella by recruitment of ATG16L1.

Major comments:

1. The authors make an unfortunate and confusing choice of wording in the title and the text of "autophagy being recruited" to damaged early endosomes. A protein can recruit another protein but it can not recruit a process or pathway to a membrane.

In the title we use the term "target". In the text, we now avoid the expression "recruiting autophagy".

2. The authors conclude that Rabaptin5 may have a role in autophagy directed to damaged early endosomes. The conclusion that Rabaptin5 binds FIP200 and ATG16L1 are convincing. The main issue is however in identifying what sort of process they are following. They have shown WIPI2 and LC3 can be recruited to early endosomes after 30 min chloroquine treatment but there is no data to explain the consequences of the binding of these proteins. They do not provide proof that canonical autophagosomes are formed which engulf and remove the damaged endosomes, nor do they show that the recruitment of WIPI2 is to a single membrane (presumably damaged early endosomes) which would be a non-canonical pathway. They often use the terminology "chloroquine-induced autophagy" (see Figure 4) but have virtually no proof they have induced either canonical or non-canonical pathways in their experiments. The only evidence they provide that there is some alteration in a membrane-mediated event is increase in lipidation of LC3 in Figure 6.
The authors must follow either an early endosome protein or cargo to demonstrate lysosome-mediated degradation indicative of autophagy, or demonstrate the process is a variation on non-canonical autophagy.

We analyzed transferrin receptor levels with and without CQ to test degradation of an early endosomal marker protein. Since CQ inhibits autophagic flux, this assay may not be very sensitive. Nevertheless, we found a significant reduction of ~15% and ~30% after overnight incubation with CQ in parental HEK293 cells and in Rbpt5-KO cells re-expressing wild-type Rabaptin5, resp., but no reduction in Rbpt5-KO cells expressing the Rabaptin5-AAA mutant defective in binding to ATG16L1. These results were included as Appendix Figure S5D and E of the revised manuscript.

2. There are concerns about the replicates done for many experiments in particular the co-immunoprecipitations which are not quantified (Figure 1 and 5).

We have now included quantitation of the immunoblots, either as graphs in the figures or as numbers in the legends.

3. The rescue experiments, even if done with stable cells lines made in the parental HEK293 cell line should be viewed with caution because of the very different amounts of Rabaptin5 (see Figure 6A). The overexpression of Rabaptin5 has not been well studied and comparisons with the mutants are therefore preliminary (Figure 6F and G).

Fig 6A shows that Rabaptin5 levels are similar except for +Rbpt, where they are higher, and R-KO, which has none. Additional Rabaptin5 seems not to significantly enhance early WIPI and ATG16L1 colocalization.

4. Conclusions about the role of the ULK complex, or ULK1 versus ULK2, should be expanded by studying the activity of the complex (phosphorylation of ATG13 for example) in order to make the conclusions more significant.

We consider analysis of kinase activity to be beyond the scope of this study. However, we have expanded the analysis to ULK2 knockdown and ULK1/2 double knockdown. As now shown in Figure 4C and D, ULK2 knockdown and even more so the ULK1/2 double knockdown reduced CQ-induced WIPI2 and LC3 puncta, indicating that CQ induction of autophagy depends to a large extent on ULK2.

Minor comments:

1. Much of the labelling in the immunofluorescence images is not visible even on the screen version.

We were cautious to have the signals within the dynamic range of the image, but we have now carefully enhanced particularly the red signals for better visibility.

2. The LC3-lipidation experiment (Figure 6D) should be re-analysed by normalization to the loading control. The result may be significantly different and is open to re-interpretation. The quality of this western blot is also very poor.

Quantitation was based on the ratio between LC3B-I and -II or the percentage of II of the total, always within the same lane and therefore largely independent of loading.

Reviewer #3 (Significance (Required)):

This manuscript topic fits into the field of study of canonical versus non-canonical autophagy. This literature is best described as "LAP" first discovered by Doug Green, (Sanjuan in 2009) but more recently as a phenomena induced by monesin, and viral infection amongst others. Most relevant to this study are the references (in the text) by Florey (Autophagy 2015), Fletcher (EMBO J, 2018) and others. However, this manuscript fails to cite and consider the critical findings in a key study published by Lystad et al., Nature Cell Biology 2019, which examines the role of ATG16 in both canonical and non-canonical autophagy. The current study if placed into the context of the Lystad study would have significantly more value, and potentially make the findings more significant.

Lystad et al (2019) provided evidence that the WIPI binding sequence 207–230 in ATG16L1 is required for canonical autophagy, but not for LAP-like autophagy, and that deleting the β insert in full-length ATG16L1 reduces non-canonical LC3 lipidation, while it does not affect canonical autophagy.
We already show that knockdown of WIPI2 prevents localization of ATG16L1 to Rabaptin5-positive CQ-damaged endosomes (Figure EV3C and D). Following the reviewer's suggestion, we have now also analyzed the ability of ATG16L1α to be recruited to CQ-induced enlarged endosomes and to colocalize with transferrin receptor in wild-type and Rabaptin5-AAA mutant cells. We found that ATG16L1α is recruited as efficiently as the β isoform with wild-type, but not with the Rabaptin5 mutant defective in binding to ATG16L1. These results support canonical autophagy and are included in Figure EV4B–D.

Furthermore, the short chloroquine treatments used here could be of interest to the field if using the cited study of Mauthe et al., (which very clearly defines the effect of chloroquine after long (5 hrs treatment)) the authors would to revisit and repeat some of the key experiments in order to demonstrate the effects of 30 minute treatment. Does such short treatment block fusion? Does it affect the pH of the acidic compartments? Does it inactivate the endocytitic pathway? As the manuscript stands the lack of this understanding of the effect of chloroquine at short times, makes the observations difficult to be place into any biological context.

- Mauthe et al (2018) analyzed lysosomal acidity upon CQ or BafA1 treatment from 30 min to 5 h using LysoTracker and found a significant increase for CQ and reduction for BafA1 treatment already at 30 min. (We can reproduce that CQ does not neutralize lysosomes at 30 min and 5 h in HEK cells.)
- They showed a gradual reduction of EEA1 puncta area from 30 min to 5 h (not yet significant after 30 min).
- They also show additivity of LC3 lipidation with CQ and BafA1 after 2 and 5 h, indicating different mechanisms of blocking autophagic flux. Our experiments shown in Appendix Figure S3A confirm this in our cells for 30 min.
- Endocytic transport of EGF and phosphorylation of EGFR required preincubation for 1 h.
- Other experiments (LDH sequestration, total proteolysis, RFP-LC3/Lamp2 colocalization) were done after 2 and 5 h and showed gradually increasing effects, sometimes not yet significant after 2 h. At 30 min the effects will be even smaller.
- Importantly, since LC3 activation/recruitment only starts at ~30 min, effects on autophagosome fusion with lysosomes cannot be investigated at these early time points. Consistent with this, when colocalizing p62 with Lamp1 after 30 min and 5 h of CQ or monensin treatment, a significant reduction was only observed after 5 h.
Overall, our experiments at 30 min of treatment describe early events of recruitment of autophagy components to early endosomes not affected by inhibition of flux or indirect effects of CQ/monensin treatment, while those at 150 min are covered by the study by Mauthe et al.

This reviewer has expertise in autophagy, autophagosome formation and is familiar with the areas of endocytosis and infection.

Referee Cross-commenting

I think a major concern about the manuscript which is present in all reviews is the lack of clarity about what type of membrane LC3 is added to- is this the damaged endosome or a forming autophagosome? This leads to the question of what type of process is being observed here? non-canonical versus canonical autophagy.

Dear Prof. Spiess,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you can see, the referees find that the study is significantly improved during revision and recommend publication after some remaining minor issues have been addressed. Please also provide a point-by-point response to these.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please reduce the number of keywords to 5.

- Appendix figure legends:
S2B: '*P<0.05' is not part of the figure panel and should thus be removed from the legend.
S2B: please define the 'CQ' and 'MRT' treatment in the legend
S3E: please remove ***P<0.001 from the legend as it is not part of the graph.
S4A: please define the difference between the open and closed arrowheads. Please change "Bar" to "Scale bar"
S5A, B: please define the scale bar in the legend.
S5D, E: please provide a legend

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- Figure callouts: Please add callouts to Fig. 1H, Fig. 2E+K and to the panels of Appendix Figs. S1,S2 + S4.

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- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the Abstract. Could you please review these as well.

- Please also take a look at the attached 'synopsis' form and the small changes I made to the text you supplied.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In their revision, the authors extensively looked at distinguishing single membrane LC3 lipidation from canonical autophagosome targetting by using genetic tools. Overall, the authors were able to clarify most of my previous concerns. I only have few remaining comments.

Figure 5: Overall, the contrast in this image appears to have been adjusted quite extensively. More specifically, the binding between FIP200 and delWD40 (Fig. 5E&E') is not necessary (I just requested the clarification of the delWD40 construct) and could be moved to the supplementary data or omitted as this manuscript is sufficiently long. However, the quantifications of newly added Fig 5G and 5H should be included along the western blot data and not just the figure legend. I am unclear what does "reduced to 1.5 +/-1.2%" means as indicated in the legend.
The binding of the Rabaptin-5 AAA mutant to FIP200 does seem reduced by eye and should be also quantified. The involvement of FIP200 in ATG16L1 binding to Rabaptin-5 should not alter the overall conclusions of this manuscript, but should nevertheless be clarified.

Is transferrin receptor a specific marker of early endosomes? It certainly does localise at early endosomes but can also be found at recycling endosomes as many other receptors. This is mainly a comment on the response to the reviewers. Figure 2A shows some transferrin receptor positive structures that do not co-localise with Rabaptin-5.

The term "endosome-phagy" have been previously used to describe the targeting of endosomes by autophagy in a manuscript cited by the authors and published in the same journal (Fraser et al, 2019). As the authors appear to observe a similar pathway, the term could be included to describe the targeting of early endosomes in their system.

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1. page 9, break not brake

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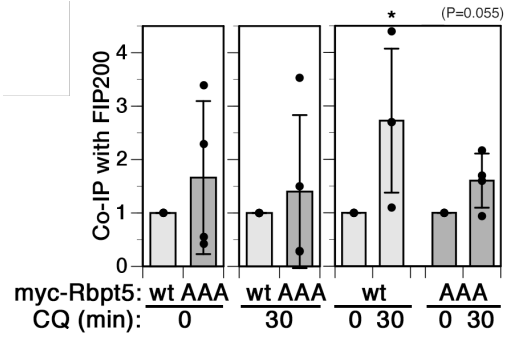
The experiments for Fig. 5E and E' were suggested by Reviewer 2.
Quantitation of immunoblots as in Fig. 5G showed coimmunoprecipitation of ATG16L1 with mycRabaptin5-AAA to be only 1.5% of that with wild-type myc-Rabaptin5. No graphical presentation is required for this result, but we are happy to add it, if you think it is useful.
Triggered by the suggestion to move Fig. 5E and E' to the Appendix, we propose to move both Fig. 5E and E' and Fig. 5H to a new Appendix Fig. S5, since both are controls to test whether there are unexpected indirect effects of a mutation on a binding site elsewhere in the protein.

The binding of the Rabaptin-5 AAA mutant to FIP200 does seem reduced by eye and should be also quantified. The involvement of FIP200 in ATG16L1 binding to Rabaptin-5 should not alter the overall conclusions of this manuscript, but should nevertheless be clarified.

Quantitation of H shows no significant change between co-immunoprecipitation of Rabaptin5 wild-type vs. AAA with FIP200 after 0 or 30 min CQ treatment, while there is an increase upon CQ treatment for both wild-type and AAA.



However, since the values scatter a lot between experiments (probably because of transient transfection), we suggest not to include them and remain with a qualitative conclusion that, as expected, the AAA mutation does not abolish interaction of Rabaptin5 with FIP200.

Is transferrin receptor a specific marker of early endosomes? It certainly does localise at early endosomes but can also be found at recycling endosomes as many other receptors. This is mainly a comment on the response to the reviewers. Figure 2A shows some transferrin receptor positive structures that do not co-localise with Rabaptin-5.

We agree that transferrin receptor is also present in recycling endosomes. The Rabaptin5-positive CQ-enlarged endosomes are always positive for Rabaptin5 and vice versa. Transferrin receptor was used to differentiate early endosomes from late endosomes and lysosomes.

The term "endosome-phagy" have been previously used to describe the targeting of endosomes by autophagy in a manuscript cited by the authors and published in the same journal (Fraser et al, 2019). As the authors appear to observe a similar pathway, the term could be included to describe the targeting of early endosomes in their system.

We added the term as suggested in the discussion.

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As suggested we performed qPCR to demonstrate significant knockdown of ULK2 mRNA. The missing siRNA was added to the Methods.

Minor points:
1. page 9, break not brake
Corrected.

Referee #3:

The authors made great efforts to address my concerns and questions (as well as the other reviewer's concerns). I still think that a mutant of Salmonella that is unable to induce damage will be good although I understand the limitations. I recommend publication in EMBO Reports.

Prof. Martin Spiess
University of Basel
Biozentrum
Klingelbergstrasse 70
Basel CH-4056
Switzerland

Dear Martin,

Thank you for sending the further revised files. I am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Corresponding Author Name:	Martin Spiess
Journal Submitted to:	EMBO Reports
Manuscript Number:	EMBOR-2021-53429V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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 $\chi 2$ 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen empirically. Experiments were replicated and tested independently at least 3 times, No data were excluded from the analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or measurements were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were randomly assigned to treatment.
For animal studies, include a statement about randomization even if no randomization was used.	NA. No animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Images not analyzed automatically were, when critical, taken in a blinded manner. When counting Salmonella colonies, quantitation was performed by an uninformed collaborator.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. The Student's t-test (two-tailed, unpaired) has been used to compare the means of two groups, while One-way ANOVA was the test used to compare the mean of three or more groups.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
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http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies are listed in with catalog numbers, providers, applications and dilutions in Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mycoplasma tests were regularly performed. HeLa cells were recently confirmed by commercial Cell line typing analysis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. 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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. 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.	NA
17. 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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All data needed to evaluate the conclusions in the paper are present in the paper and/or the Expanded View.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA