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ER-plasma membrane contact sites contribute to autophagosome biogenesis by regulation of local PI3P synthesis

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Thank you for submitting your manuscript for consideration by the EMBO Journal.

Your manuscript had been under peer-review at a different journal before, and you provided a revised version and a point-by-point response to the issues previously raised. I have now received input from an arbitrating referee on your work and your response to the previous referees. As you will see below, the arbitrating referee appreciates your responses. However, this referee notes a few issues that still need your attention.

I would thus like to invite you to provide a revised version, addressing point 1 and the minor point raised by this referee. Regarding point 2 mentioned by this referee, I would like to suggest to remove the data from suppl figure 9 as these are inconclusive and not adding much at this stage.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORT

Referee #1:

Overall I think the authors have responded well to the comments from the reviewers, but there are some issues.

Major points:

1. The conclusion that E-Syt depletion selectively affects the autophagosomal pool of PI3P is not sufficiently supported by the data in Fig 6a-c. Firstly, the strong nuclear localization of PI3P in starved cells is unaccounted for and confounds the quantification. Secondly, the assumption that EEA1 is present on all PI3P-positive endosomes is false since PI3P is also abundant on EEA1-negative endosomes such as late endosomes and recycling endosomes.

2. Suppl.Fig. 9 reports some potentially very important findings, but the data here are not conclusive. Firstly , the images in b are of too low quality to allow meaningful quantifications. Secondly, the counting of LC3-positive structures by itself does not reveal whether autophagic flux is affected. Given the importance of this issue, the authors need to do a proper flux analysis as recommended by Klionsky et al, Autophagy, 2016.

Minor point:

The acronyme "PI3KC3" for the class III PI 3-kinase complex is confusing since it strongly resembles "PIK3C3", the gene/protein name for human VPS34.

AUTHORS' RESPONSE TO THE PREVIOUS REFEREES:

- *interesting results, but claims not convincingly supported*
- *more insight into E-Syt / ER-PM contact mediated PI3P synthesis regulation needed.*

We are pleased that this reviewer found our results interesting. Based on this reviewer's comments, we have analysed more deeply the mechanisms by which E-Syts and the ER-PM contact site machinery participate in the regulation of autophagy-related PI3P synthesis. We now show that VPS34 enzymatic activity is not altered by ER-PM contact sites destabilization, whereas activities of Beclin1 and ATG14L, the two specific regulators of VPS34 in autophagy, are affected. Our new data show that E-Syt2 protein interacts with the ER-located VMP1 protein, which was previously shown to have a major role in autophagy initiation. This E-Syt2/VMP1 interaction drives the targeting of Beclin1 to E-Syts ER subdomains to ensure local PI3P synthesis and promotes autophagosome biogenesis at ER-PM contact sites. These results are shown in new Fig 7 and new Fig 9 and Appendix Fig S4.

- *autophagosome biogenesis occurring preferentially at ER-PM contacts needs more experimental data*
- *visualization of the earliest steps of autophagosome formation over time needed to clarify role of E-Syts*

To provide compelling evidence for autophagosome biogenesis events at ER-PM contact sites, we used several additional approaches. We now clearly demonstrate that autophagosome assembly at ER-PM contact sites are bona-fide events observed soon after autophagy induction (5 to 15 min of starvation). We now show co-localization of E-Syts with LC3 at ER subdomains by super-resolution microscopy (new Fig 3A). Moreover, we observed ER-associated pre-autophagosomes/autophagosomes in the immediate vicinity of PM by electron microscopy (new Fig 3C). Quantification of these structures revealed that autophagosome biogenesis at ER-PM contact sites represent approximatively 33% of total autophagosomes after starvation, while autophagosomes emanating from ER-mitochondria contact sites represent approximatively 25% of the total autophagosomes (new Fig EV3D). Similar results were obtained by using DFCP1, a pre-autophagosomal marker (as suggested by this reviewer): We now show association of DFCP1 structures with E-Syts markers by video microscopy (new Fig 4). The percentages of pre-autophagosomal structures at ER-PM and ER-mitochondria contact sites observed by video microscopy were around 35% and 28%, respectively (new Fig EV3A-C). Altogether, our new data, obtained by light, video, and electron microscopies,

clearly establish the importance of ER-PM contact sites during the formation of autophagosomes; these sites are similar, in terms of number, to previously described ER-mitochondria contact sites.

- *how is PI3P synthesis or localization affected by E-Syt levels or ER-PM contacts, is it a direct effect*

We now precisely address how E-Syts participates in PI3P synthesis regulation, as briefly mentioned in our introducing reply to this reviewer's comments. We show that E-Syts deficiency (which destabilizes the ER to PM tethering) has a strong effect on Beclin1 and ATG14L stability during autophagy-inducing conditions (new Fig EV6A) but little effect on VPS34 turnover. We also quantified the autophagy-related PI3P (by indirect fluorescence) and noticed that in cells deficient in E-Syts levels of PI3P were reduced compared to controls (new Fig 7A-C). Using an in vitro PI3P synthesis assay, we found that VPS34 enzymatic activity was not modified by the absence of E-Syts (new Fig 7D-E), whereas the stability of the PI3KC3 complex was altered. At the molecular level, the recruitment of the PI3P-binding protein WIPI2 to ER-PM contact site zones was abolished in cells lacking functional E-Syts (new Fig 7F), and this was accompanied by a decrease in the amount Beclin1, ATG14L, VMP1, and WIPI2 at these sites (new Fig 7G-J). Overexpression of E-Syt2, E-Syt3, or E-Syt2 and E-Syt3, which artificially boosted the numbers of ER-PM contact sites (new Appendix Fig S1), lead to an increase of VMP1, Beclin1, and ATG14L protein stabilities but had no effect – or little effect – on VPS34, VPS15, and ATG13 stabilities (new Fig 9C). Finally, we now demonstrate that E-Syt2 interacts with the ER-located autophagy regulator VMP1, which is known to stabilize Beclin1, and we show, by co-immunoprecipitation and confocal and video microscopies, that the three proteins are tightly associated in the early stages of autophagy induction at ER-PM contact sites (new Fig 8 and new Figure 9A, B, and C). We also show that the VMP1-E-Syt2 association and dissociation are key events in autophagosome biogenesis, since an autophagy-incompetent VMP1 mutant that is unable to bind Beclin1 (namely ΔATGD-VMP1) is permanently associated with E-Syt2, as we demonstrated by co-immunoprecipitation and fluorescence microscopy (new Appendix Fig S4).
Taken together, our results suggest a close interplay between E-Syts machinery and the well-established regulators of PI3KC3, such as Beclin1, via the VMP1 protein. We now propose a model that in an autophagy-inducing stress situation, the mobilization of cortical ER near the PM (new Fig 1 and Fig EV1) directs a specific pool of PI3KC3 complex to the ER-PM domains via the E-Syt2/VMP1/Beclin1 interaction. Once there, the complex is engaged in local PI3P synthesis (detected by PI3P labelling as well as recruitment of DFCP1 and WIPI2 proteins), which leads to local autophagosome biogenesis. Then, the complex dissociates (new Fig 9D), and VMP1 travels with the autophagosomal membrane, while E-Syt2 remains at ER membrane facing the PM. Importantly, our results indicate that E-Syts are directly involved in local PI3P synthesis in response to autophagy induction.

- *how often does autophagosome production occur at ER-PM contacts and how often at ER-mitochondria contacts (and how often at other locations)*

This is indeed an important point raised by this reviewer. As described in our reply 2. we now show that the biogenesis of autophagosomes at ER-PM contact sites accounts for approximatively one third of total autophagosomes, which is in the range observed by electron and light microscopy (Fig 4 and Fig EV3).

- *more immuno-EM or tomography examples are necessary as well as quantification to support autophagosome formation at ER-PM*

We thank the reviewer for the suggestion that we use immunological techniques to visualize autophagosome biogenesis at ER-PM contact sites. We were not able to perform tomography to visualize autophagosome formation near ER-PM contact sites, but we now show the presence of phagophores and autophagosomes in the immediate vicinity of ER and PM contact zones by electron microscopy (new Fig 3C), and we improved our analysis of E-Syts and LC3 co-distribution using super-resolution microscopy (new Fig 3A). Moreover, as recommended by this reviewer, we quantified our electron microscopy data, revealing that approximatively

33% of total autophagosomal structures were associated with ER-PM contact sites (new Fig EV3).

- *is it tethering or some activity of E-Syts that is required? This might be resolved by using E-Syts mutants that cannot bind lipids or by using an artificial tether.*

This was a very helpful suggestion. Based on this reviewer recommendation, we now show that overexpression of wild-type E-Syt2 and E-Syt3 rescue the effects of E-Syts deficiency using based on analyses of autophagy readouts (such as LC3 lipidation) and PI3P-related phenotypes (such as WIPI2, VMP1, Beclin1, and ATG14L proteins). In contrast, overexpression of tethering-defective mutants of E-Syts proteins (lacking the C2C domain) failed to rescue these phenotypes (new Fig 7G-J). These experiments, based on fluorescence analyses of WIPI2 protein puncta formation as well as biochemical analyses of the stabilities of autophagosomal machinery regulators, now clearly show that the tethering functions of E-Syts are required for local autophagosome assembly.

- *Although the work is potentially important and for the most part carefully done, there are significant issues of omission and interpretation that could change the conclusions for both approaches outlined above-they will need to be rigorously addressed.*

We would like to thank this reviewer for finding that our work is "*potentially important and for the most part carefully done*", and we address all suggestions and comments with specific and robust experiments that, we think, will improve the overall interpretation of our results. Moreover, we have added a substantial amount of new data that directly implicate regulatory complexes located at ER-PM contact sites in autophagosomal PI3P pool synthesis via the E-Syt2/VMP1/Beclin1 crosstalk (new Fig 7, new Fig EV6, Fig 8 and Appendix Fig S4).

- *the starvation protocol used leads to cell spreading irrespective of amino acid presence. Controls are thus needed with amino acid supplementation and with mTOR inhibition to rule out effects being due to simplified growth conditions.*

We would like to thank this reviewer for suggesting these experiments. We now show that E-Syt2 protein expression is induced by mechanical stress, serum starvation, and mTOR chemical inhibition by Torin1 (new Fig EV1). These data provide further evidence that E-Syt2 responds to multiple autophagy-mediated stressors.

- *effect of E-Syt downregulation on autophagosome number could be due to the observed reduction in ATG14 and Beclin levels and thus independent of ER-PM contact sites. This needs to be explained and better support for a role of E-Syts in autophagosome number regulation provided. What is the basis and significance of the reduction in the levels of ATG16L1 and ATG5-12 in the triple E-Syt KO cells?*

This reviewer raised an important question regarding the crosstalk that could exist between E-Syts/ER-PM contact site machinery and autophagosome-assembly regulators. The stability of several ATG proteins is linked to their timing of dissociation from the autophagic structures (Koyama-Honda et al., *Autophagy* 2013). The differences in ATG16L1, ATG5-12, Beclin1, and ATG14L levels could be explained by a partial – but permanent – lack of a PIK3C3 complex stabilizing system. Indeed, we now describe the molecular link that connects the ER-PM tethering complexes and the autophagosomal machinery. We show that E-Syt2 protein interacts with the ER-located VMP1 protein driving the targeting of Beclin1 to E-Syts ER subdomains to ensure local PI3P synthesis and to promote autophagosome biogenesis at ER-PM contact site domains. These results are described in the new Fig 8 and Appendix Fig S4.

- *relationship of LC3 puncta to ER and to PM based on some type of proximity measurement must be provided in quantitative terms.*

This was a very interesting suggestion. When studying autophagosome biogenesis, however, it is potentially misleading to quantify only LC3 puncta since LC3 is a marker for pre/mature and autophagosome structures. Similar experiments have been done using the omegasome-associated DFCP1 protein, which is considered a good marker for autophagosome biogenesis

location analysis. We now show that E-Syt/LC3 co-distribution was often associated with DFCP1 and ER-marker Sec61β, as shown by confocal microscopy, 3D acquisition, and video-microscopy (new Fig 4). Moreover, we quantified DFCP1 co-distribution with the E-Syt2/ER interface (for ER-PM contact sites) and with the TOM20/ER interface (for ER-mitochondria contact sites) to show that approximately 30% and 25% of DFCP1 structures were associated with E-Syt2 and TOM20, respectively (new Fig EV3A-C). Importantly, similar results were obtained by direct observations of autophagic structures by electron microscopy in cells stained for ER (new Fig EV3D). This new data indicates that the assembly of autophagosomes at ER-PM contact sites accounts for at least one third of total autophagosome assembly, a percentage very similar to that we observed for ER-mitochondria-associated autophagosome biogenesis. Finally, we now show LC3 and E-Syt2 co-distribution by super-resolution microscopy analyses (new Fig 3A) and demonstrate the presence of pre-autophagosomal autophagic structures, such as phagophores, in ER-PM contact zones by electron microscopy (new Fig 3C).

- *effects of Syt2 and Syt3 overexpression on ER-PM contact sites must be shown directly in the paper and not rely on the previous De Camilli data.*

As suggested, we now provide data that clearly show that overexpression of E-Syt2 and E-Syt3 proteins induce a strong recruitment of ER in the plasma membrane vicinity (new Appendix Fig S1).

- *there are EEA1-negative early endosomes that still contain PI3P. Use of EEA1-negative FYVE probe to identify autophagy-related PI3P pools must be supplemented with analyses of WIPI2 and DFCP1 puncta upon E-Syt overexpression or downregulation.*

This is indeed an important point. We previously overcame this issue by using a different marker of the endosomal membrane (new Fig EV6C). We now show that WIPI2 recruitment to cortical ER upon autophagy induction was abolished in E-Syts depleted cells (new Fig 7f). Overexpression of wild-type E-Syt2 or E-Syt3 rescued this phenotype, and we now report that tethering mutants of both E-Syts (lacking the C2C domain) were not able to restore proper WIPI2 targeting (new Fig 7G-I).

- *effects of starvation on E-Syt expression (Fig 1E) must be tested for significance, and also tested with other incubation conditions*

We now show, thanks to this reviewer, that E-Syt2 turnover is affected in additional autophagy-promoting situations (new Fig EV1).

- *live imaging support for PM and ER coming together to generate autophagosomes is needed.*

We thank the reviewer for this excellent suggestion. As this reviewer is probably aware, there is no – to date and to our knowledge – reliable "plasma membrane" marker that can be used in live imaging, because of the high endocytosis and recycling activity occurring at the cell surface. However, as suggested by this reviewer, we were able to show, by video microscopy, DFCP1 association with E-Syts domains upon autophagy induction (new Fig 4C) as well as transient association of Beclin1 and VMP1 with E-Syts proteins in the same conditions (new Fig 9B). These new pieces of data support our hypothesis that the PM and ER contact sites generate autophagosomes.

- *it is unclear whether autophagosomes are preferentially formed at the ER-PM contact site and what is the essential difference between the ER-associated and ER-PM-associated autophagosome formation processes. The study is too preliminary to support the authors' hypothesis.*

We thank this reviewer for the useful comments. We agree that our previous version of the study was too preliminary. As detailed below, we now add a substantial amount of new data that unravel the role of the ER-PM contact sites in autophagy-related PI3P synthesis and thus in autophagosome biogenesis. Notably (as detailed in point 3 below), we now report that E-

Syt2 protein interacts with the ER-located VMP1 protein which is known to play a major role in autophagy. This E-Syt2/VMP1 interaction drives the targeting of Beclin1 to E-Syts-containing ER subdomains to ensure local PI3P synthesis and promotes autophagosome biogenesis at ER-PM contact sites. Our new mechanistic data are reported in new Fig 7 and 9 and new Fig 8 and Appendix Fig S4.

- *does autophagosome formation take place preferentially at ER-PM contact sites?*
- *triple knockdown of E-Syts reduces the total number of LC3 puncta by only 30% (Fig. 6D). Autophagic flux is not determined in these cells, and off-target effects of the triple knockdown are not ruled out by rescue experiments.*
- *even if autophagosomes can be generated at the ER-PM contact site, it is not essential and may not represent a major contribution.*

First, we would like to apologize if some of our results were not clearly reported and/or highlighted. For example, the question of autophagic flux raised by this reviewer was previously addressed but the data presentation has been revised (new Fig EV5). Second, we did not intended to claim that autophagosome assembly occurred "preferentially" at ER-PM contact sites, since we do observe autophagosome biogenesis elsewhere in the cell, as expected. Rather, we sought to shed light on the possibility that during stress (addressed now in serum starvation, mTor inhibition, and mechanical stress situations), a significant fraction of autophagosomes are formed at ER-PM contact sites. Importantly, we now report, in full starvation conditions, that approximately 30% of total autophagosomes are formed at ER-PM contact sites and approximately 25% come from ER-mitochondria contact sites.
Quantifications of electron microscopy analyses (new EV3D) and of co-distribution of DFCP1 (a marker of early stage of autophagy) with E-Syts or mitochondria markers (new Fig EV3A-C) were performed.
Moreover, as suggested by this reviewer, we now rule out off-target effects of E-Syts siRNA-mediated knock-down by rescue experiments: We show that overexpression of wild-type E-Syt2 and wild-type E-Syt3 rescue the effects of siRNA-mediated inhibition of E-Syts expression on autophagy readouts (such as LC3 lipidation and WIPI2 puncta formation). Overexpression of tethering-defective mutants of E-Syts proteins (lacking the C2C domain, responsible for the ER to PM tethering) failed to rescue these phenotypes (new Fig 7G-J). These experiments, based on fluorescence analyses of WIPI2 recruitment and stability of autophagosomal machinery regulators now clearly show that the tethering functions of E-Syts are required for local autophagosome assembly and that this assembly depends on specific PI3P synthesis. Together with our new data show that the close interplay of regulation of E-Syts proteins and PI3KC3 activity occurs via the ER protein VMP1 (new Fig 9 and new Fig 8 and Appendix Fig S4). Our results indicate that about one third of autophagosomes are indeed specifically assembled at the cortical ER facing the plasma membrane and that this assembly is under the control of E-Syts proteins.

- *how do ER-PM contacts contribute to biogenesis of autophagosomes?*
- *can the reduced expression of BECN, ATG14L, and VPS34 fully explain the defect? Does the ULK1 complex, an upstream factor, accumulate there?*
- *there is no evidence that the EEA-negative PI3P-positive structures observed in triple KD cells are indeed involved in autophagosome formation (Fig. 7).*
- *it is premature to conclude that the ER-PM contact plays a major role in autophagosome formation through local regulation of the PI3P content.*

As stated above, we now provide robust mechanistic data that clarify the role of ER-PM contact sites in autophagy-associated PI3P synthesis. As suggested by this reviewer, we have now analyzed the behavior of ULK1 protein in cells deficient in E-Syts and showed that ULK1 recruitment to ER was not affected by absence of ER-PM contact site regulators (new Fig EV6B-C), as expected based on our previous results showing that members of the ULK1 complex were not affected lack of E-Syts proteins (new Fig EV6A).
As noted by this reviewer, we observed alteration of Beclin1 and ATG14L stability during autophagy induction and this lead us to investigate the relationship that exists between E-Syts and regulators of PI3KC3. We now show that enzymatic activity of VPS34 is not directly modified by absence of E-Syts proteins (new Fig 7D-E). However, overexpression of E-Syts did result in increased stability of several proteins of the PI3KC3 complex, such as ATG14L and

Beclin1, as well as external regulators such as the VMP1 protein (new Fig 9C). In contrast, overexpression of E-Syts did not alter levels of ATG13 or ER-protein CLNX. Based on these observations, we questioned the role of the VMP1 protein, since this ER-autophagy regulator was previously reported to be essential for Beclin1 recruitment to the future autophagosomal membrane. We indeed observed strong co-localization of Beclin1 and VMP1 soon after autophagy induction. Interestingly, a consistent amount of Beclin1/VMP1 association was associated with the cortical ER in the vicinity of the plasma membrane in co-distribution with E-Syts proteins (new Fig 8).
We finally show that E-Syt2, VMP1, and Beclin1 are dynamically associated, as detected by co-immunoprecipitation and video microscopy (new Fig 9A, B, and D). Interestingly, this association diminished by 30 minutes after autophagy initiation (new Fig 9D), a phenomenon that was supported by our observations (and already reported for VMP1 and Beclin1) showing that E-Syts proteins remain associated with the ER membrane after autophagosome formation (vesicular LC3/E-Syt2 was only observed at the ER membrane), while VMP1 can travel with LC3 vesicles and Beclin1 is recycled in the cytosol. These observations were confirmed by our demonstration of a permanent association of E-Syt2 and ΔATG-D VMP1 (a mutant of VMP1 that blocks autophagosome biogenesis and is unable to bind to Beclin1); new Appendix Fig S4). These new data support our hypothesis that ER-PM domains that are the sites of PI3P synthesis are locations of autophagosome assembly.

- *co-localisation experiments are not convincing. Co-distribution and vicinity to the PM should be analysed. Time-lapse movies will be of help to show that these structures are indeed associated with each other.*

We prefer to use the term "co-distribution" rather than "co-localization". The former is based on biological distribution of molecules in a given area (for example, membranes); the latter implies the detection of "white" or "yellow" signals. Indeed, from a biological point of view and by using confocal or high-definition wide field microscopy settings, we do not expect a total co-localization pattern between a signal coming from a vesicular marker (such as LC3) and reticular marker (such as Sec61 (for ER) or E-Syts (for regions of the ER engaged in contact sites with PM)). As previously mentioned we now provide quantifications of the DFCP1 co-distributions with E-Syt3 and Sec61 proteins or with TOM20 and Sec61 proteins, which clearly show that approximately 30% of pre-autophagosomes observed in a given cell are associated with ER-PM contact sites (new Fig EV3A-C). These data, obtained by manual quantification of fluorescent signals, were confirmed by electron microscopy studies (new Fig EV3D).
As suggested by this reviewer we have now evaluated the co-distribution of E-Syts and autophagic markers by light microscopy: We provide super-resolution microscopy data showing without doubt that part of LC3 staining is associated with E-Syt-positive ER membranes upon autophagy induction (new Fig 3A).
Finally, as suggested by this reviewer, we now show, by time-lapse microscopy, the DFCP1 association with E-Syts-containing domains (new Fig 4C) upon autophagy induction. We also demonstrate the transient association of Beclin1 and VMP1 with E-Syts proteins in the same conditions (new Fig 9B).

- *LC3 can be present on both phagophores and autophagosomes, and is thus not an appropriate marker to detect forming autophagosomes (e.g. Fig. 6D and Fig. 2). DFCP1 should be a better marker for the site of autophagosome biogenesis.*

We appreciate this suggestion, and we have used DFCP1 in many new experiments (new Fig 4 and EV3) as well as WIPI2 (new Fig 7).

- *Fig. EV2D: Showing only one gold particle for each signal is not sufficient.*

We now provide uncropped images showing large fields with several gold particles (new Fig EV2D).

- *scale bars in magnified images would also be helpful.*

Scale bars are now shown in magnified images.

- *how is the "cortical ER PM occupancy (%)" calculated?*

For the quantification of cortical ER in HRP-stained Epon sections, the total length of plasma membrane and the length of the multiple HRP-positive cortical ER segments were measured by iTEM software on acquired micrographs of HeLa cells for each of 20 uninterrupted cell profiles showing the ER-PM contact phenotype in each of three experimental replicates. All data are presented as the mean percentage.

- *Fig. 6D: "Peripheral" and "perinuclear" should be defined.*

We clarified the both definitions in the legend of the figure.

SECOND ROUND OF PEER-REVIEW:

- *manuscript improved and more evidence for a fraction of autophagosome formation occurring at ER-PM contacts.*
- *data still not sufficient to support that) ER-PM contact sites are required for autophagosome biogenesis, and that E-Syts play a direct role in mediating PI3P synthesis by the PIK3C3*

We would first like to thank this reviewer for acknowledging the improvement of our manuscript, and for underlining that we now show better evidence that "*a significant fraction of autophagosome formation occurs at ER-PM contacts*". As this reviewer requested, the revised manuscript provides additional evidence to support claims 2 and 3. Regarding concerns about "the requirement of *ER-PM contact sites for autophagosome biogenesis and the direct involvement of E-Syts in PI3P synthesis*", we agree that some of our conclusions were misworded. We have demonstrated that a subset of autophagosomes forms at ER-PM contact sites via the involvement of E-Syts proteins, which are specifically engaged in local autophagy-associated PI3P pool synthesis. We did not intend to claim that E-Syts mediate the general synthesis of PI3P on membranes, including endosomal membranes or other membranes related to autophagosome biogenesis. Therefore, we rephrased some of our major conclusions and, accordingly, we changed the title of the manuscript to: "ER-PM contact sites contribute to autophagosome biogenesis by regulation of local PI3P synthesis".

- *E-Syt knock-down reduces but does not block autophagosome formation. What important role do ER-PM contacts play in autophagosome formation and how do other locations of formation substitute for this role when ER-PM contacts are disrupted?*

The first round of revision of the paper provided, by several approaches, additional strong evidence for autophagosome biogenesis events occurring at ER-PM contact sites. This justifies and explains the sentence *"...demonstrate that ER-PM contact sites are required for autophagosome biogenesis in mammalian cells"*. However, we did not intend to state that ER-PM contact sites are the only site at which autophagosome biogenesis occurs. We reworded the sentence as follows *"... ER-PM contact sites are mobilized for autophagosome biogenesis in mammalian cells"*. Notably, and as requested by this reviewer, we monitored over time (by video microscopy) and by the use of early autophagy markers (by super-resolution microscopy) these events and quantified them by comparison with events occurring at other ER-membrane contact sites. The pre-autophagosomal structures observed by video and light microscopy at ER-PM and ER-mitochondria contact sites were 35% and 28% of total, respectively (Fig EV3), suggesting that ER-PM contact sites are at least as important as ER-mitochondria contact sites (Hamasaki, M. et al, Nature 2013; and our results) for autophagosome biogenesis.
Altogether, our new data show that ER-PM contact sites are as important as ER-mitochondria contact sites in autophagy and suggest that both tethering complexes act in coordination. Thus, the fact that E-Syts knock-down reduces but does not totally block autophagosome formation is not surprising and rather expected. We strongly believe that our set of experiments now demonstrates that ER-PM contact sites associated with E-Syts play a role in the response to different stressors, such as amino acid starvation, serum starvation,

pharmacological inhibition of mTOR and mechanical stress – all conditions known to induce autophagy.

- *the evidence supporting that E-Syts play a role in mediating PI3P synthesis is only indirect. Mechanistic insight into how, specifically, E-Syts regulate PI3P levels is necessary to definitely prove that they do.*

We respectfully disagree with this analysis. Our data show that E-Syts contribute to VPS34/Beclin1 complex stability at ER-PM contact sites by interacting with VMP1 (an ER partner of Beclin1), and we think this is a valuable result *per se*. Interestingly, a recent paper showed that VMP1 should be considered as an ER-driven contact site regulator (Tabara et al, 2016), which makes sense with our own data, and strengthens our hypothesis. Moreover, in addition to indirect evidence of the role of E-Syts in regulating PI3P synthesis (i.e., altered stability of key members of the autophagy-associated VPS34 complex, such as ATG14 and Beclin1, in E-Syts-deficient cells), we also provided direct evidence of the role of E-Syts in PI3P synthesis. By monitoring autophagy-related PI3P pool by immunofluorescence, we showed that the levels of autophagy-related PI3P are significantly reduced in E-Syts-deficient cells compared to control cells (Fig 7A-C). In addition, we have performed rescue experiments demonstrating that E-Syts are required for the recruitment of the autophagy regulator and PI3P-binding protein WIPI2 to ER-PM contact sites (Fig 7F-J). Downregulating E-Syts did not alter VPS34 enzymatic activity; however, it affected the VPS34 partners VMP1, Beclin1, and ATG14L, giving insights into local PI3P synthesis regulation. The specific mechanism will have to be thoroughly dissected in future experiments that are beyond the aims of our present paper.
As stated above, we do not claim that E-Syts regulate all PI3P synthesis but rather synthesis of a portion of the autophagy-related PI3P pool. It should be taken into account that small and transient subsets of PI3P-membranes are difficult to analyse dynamically. Indeed, we would like to remind this reviewer that PI3P is transitorily synthesized at other autophagy-related membranes and permanently at endosomal membranes. This is well illustrated by the fact that E-Syts knock-down did not affect endosomal PI3P (Fig 7A).

- *in the revised version the authors have tried to address the previous concerns, but, unfortunately, this manuscript continues to have critical issues.*
- *the authors now suggest that only approximately 30% (or 20% from Fig EV3D) of autophagosomes are formed at ER-PM contact sites in an E-Syts-dependent manner.*

Results shown in Fig EV3D indicate that, upon autophagy induction, averages of 23, 20, and 33 autophagic structures are detected per cell at ER-PM contact sites, ER-mitochondria contact sites, and cytoplasm, respectively. Thus, the proportion of autophagosomes formed at ER-PM contact sites quantified from electron micrographs is 30.3% of the total, and not 20%. These data confirmed results obtained by immunofluorescence analysis. These data suggest that ER-PM contact sites are at least as important for autophagosome biogenesis as the ER-mitochondria contact sites previously described (Hamasaki et al., Nature 2013), highlighting the importance of such tethering domains of the ER in the initiation of autophagy (see below, comment #3).

- *the authors speculate that E-Syts stabilize the PI3KC3 complex through interaction with the VMP1 regulatory protein. Why are E-Syts not important for autophagosome biogenesis in places other than the ER-PM contact site, where PI3KC3 is also essential?*

PI3KC3 and VMP1 are essential for canonical autophagy: the former for its intrinsic PI3P production ability and the latter for recruitment of the PI3KC3 complex to ER subdomains (nature of which still remains an open question) associated with autophagosome assembly (Itakura and Mizushima, Autophagy 2010). To answer this reviewer's important question, one tantalizing hypothesis is to consider that ER-driven membrane contact sites (with PM, with mitochondria, or other membranes) are local platforms for PI3KC3 recruitment. Thus, different partner proteins, such as E-Syts for ER-PM contact sites and STX17 for ER-mitochondria contact sites, might play a local role and confer a level of specificity to the early autophagy processes.

E-Syts proteins, in particular E-Syt2 and 3, are constitutive ER proteins and nearly exclusively expressed at ER-PM contact sites: therefore, they are not present at other cellular sites (Appendix Fig S1A). This is most likely the reason why these proteins participate only in autophagy events occurring at ER-PM contact sites. This highlights the novelty of our results, which shed light on how cells might cope with stress situations and adapt their response by diversifying the protein partners interacting with the "core" proteins (such as PI3KC3 and VMP1) involved in the autophagy-mediated response. It is worth noting that in our rescue experiments (see new Fig 7) the crucial role and specificity of E-Syts are clearly proven, as demonstrated by the rescue of WIPI2 puncta and PI3KC3 complex stability after re-expression of wild-type E-Syts. However, as shown in new Fig 7, the ΔC2C-E-Syts mutants (that are unable to localize to ER-PM contact sites but rather redistribute throughout the ER) were incapable of rescue of the E-Syts knockdown. This further confirmed that E-Syts tethering – and therefore proper localization at ER-PM contact sites – is required for functions in local PI3P synthesis required for autophagosome biogenesis.

- *physiological significance of autophagosome biogenesis at PM should be investigated (e.g. important for response to mechanical damage?).*

Further experiments will be necessary to address precisely and globally the "*significance of autophagosomes biogenesis at the ER-PM contact sites*". We have initiated experiments to achieve proteomic analysis and cell fractionations to isolate the pool of autophagosomes formed at ER-PM contact sites in control and E-Syts-deficient cells under different types of stress (starvation, serum starvation, mechanical flux). These time-consuming efforts go beyond the scope of the presented work, however. In our opinion, the importance of this manuscript resides in the deciphering of a novel mechanism of autophagosome generation that furthers our understanding of the importance of ER-driven contact sites in autophagy membrane dynamics. Although we agree that investigation of the mechanism of these events and of the conditions that induce autophagosome biogenesis at ER-PM contact sites should be undertaken, waiting for these experiments would significantly retard the dissemination of the relevant message of our current study. We believe that the experimental data presented in the revised version of the manuscript are broad and robust enough to indicate that autophagosome biogenesis at ER-PM contact sites mediates responses to multiple stressors and that its distinguishing mark is a rapid, location-driven response.

- *it is well established that VMP1 is essential for canonical autophagy, so the addition of the VMP1 part does not much strengthen this point.*

Although it is known that VMP1 is required for canonical autophagy, its role is still poorly understood. Interestingly, a recent paper shows that VMP1 acts as an ER-driven contact site regulator (Tabara et al., PLoS One 2016), which makes sense in light of our own data. Indeed, our work gives insights into the mechanism by which VMP1 regulates autophagy initiation – at least at ER-PM contact sites – through interaction with E-Syts proteins on the cortical ER facing the PM (as illustrated in new Fig 8). Again, this interaction could confer specificity to the autophagosomal pool forming at ER-PM contact sites and, from this point of view, VMP1 could represent another crucial protein that, like the PIK3C3 complex I, adapts the autophagy process to different requirements by interacting with different partners at different sites (and possibly at different times as well).

- *definition of the PM-ER contact site and peripheral regions remains unclear and the support for ER-PM contact site dependent events is not sufficient:*
- *E-Syts can be detected throughout the cytoplasm not only at the PM (Figs. 3A, 7F, Fig EV3A, and 8B). ULK1 (Fig EV6B) and WIPI2 (Fig. 7F) are detected mostly in the cytoplasm.*
- *In Fig. 2a, the authors state that they are looking at "the cell boundary", but they are looking at a " perinuclear region" not the boundary.*
- *The autophagic structures shown in Fig EV4A seem to be present in the cytoplasm not near the PM.*
- *In Fig. 9A, there is only a very narrow space between the nucleus and the PM. Both Becliln 1 and VMP1 appear to be present in this narrow cytoplasmic region not preferentially close to the PM.*

We apologize for this misunderstanding, probably due to a lack of clarity in our description. Several papers indicate that E-Syt2 and 3 are genuine and reliable ER-PM contact site markers, notably the seminal work of Pietro Di Camilli, who extensively characterized these proteins as constitutive tethers of ER-PM contacts (Giordano et al., Cell 2013). E-Syt3 is almost exclusively present at cortical ER and the rough ER pool of E-Syt2 is minimal. The images we show were acquired at the basal plane of the cell (mostly corresponding to cortical ER). We have added data that clearly illustrates the above described intracellular repartition of E-Syt2 protein (new Appendix Fig S1).

- *data lack quantification (e.g. Fig. EV2 and Appendix Fig S3).*

We thank this reviewer for noticing this; quantifications are now associated with these data (see new Fig EV2 and Appendix Fig S3).

- *structures shown in new Fig. 3c do not look like typical autophagosomes. Upper structure is not an autophagosome/phagophore because something is visible in the intermembrane space.*

We thank this reviewer for this suggestion. This image has been replaced with a clearer image (see new Fig 3C).

- *Fig. 5A: Autophagic flux assay is required.*

We thank this reviewer for her/his suggestion; autophagic flux analysis is now provided in experiments concerning E-Syts overexpressing cells (see new Fig 5).

- *9. Fig. 6: Why do LC3 structures accumulate at the peripheral region by BafA1 treatment? Are there lysosomes at the periphery?*

In the new Fig 6 we show an example of the LC3 immunostaining pattern and the relative quantification of LC3 puncta for E-Syts-deficient cells versus control (after BafA1 treatment to block autophagosome consumption). Quantifications are calculated with reference to control. In E-Syts-deficient cells the total number of autophagosomes was decreased compared to control, and when we differentially quantified autophagosomes located in the perinuclear versus the peripheral region, we were able to determine that this loss was due to loss from the peripheral zone, consistent with our hypothesis that only autophagosomes at ER-PM contact sites are affected in this situation. Indeed, the decrease accounted for about the 30% of the total number of autophagosomes, in agreement with our previous estimations. Thus, quantifications did not indicate an accumulation of LC3 structures at the peripheral region of the cell, but rather a decrease of LC3 peripheral structures in E-Syts-deficient cells, reflecting the alteration of autophagosome biogenesis at ER-PM contact sites.

1st Revision - authors' response 22 April 2017

Referee #1:

Overall I think the authors have responded well to the comments from the reviewers, but there are some issues.

We thank the referee for her/his positive remarks and for finding that we "responded well to the comments" from the previous reviewers.

Major points:

1. The conclusion that E-Syt depletion selectively affects the autophagosomal pool of PI3P is not sufficiently supported by the data in Fig 7A-C. Firstly, the strong nuclear localization of PI3P in starved cells is unaccounted for and confounds the quantification. Secondly, the assumption that

EEA1 is present on all PI3P-positive endosomes is false since PI3P is also abundant on EEA1-negative endosomes such as late endosomes and recycling endosomes.

This is indeed a very important point. Here we used an indirect technique to label endogenous PI3P on fixed cells via a recombinant FYVE-GST peptide followed by a fluorescent anti-GST antibody (as previously tested in Khaldoun et al. 2014, Mol. Biol. Cell.). We recently described the ins and outs of such a technique (Nascimbeni et al. 2017, Autophagy (accepted)), which is indeed less convenient than GFP-FYVE or GFP-PX domains overexpression and associated fluorescence. However, transfection of these GFP-constructs in living cells leads to alteration of both endosomal and autophagosomal pools of PI3P (our findings, Nascimbeni et al. 2017, Autophagy (accepted)), probably by artificial stabilization of PI3P membrane domains. We thus consider that, despite inherent difficulties coming along with our FYVE-GST technique (such as heterogeneous nuclear staining, as noted by this referee), this PI3P labelling method is probably the best so far, since it is aimed to be used on fixed cells, and gave an in time picture of unaltered pools of PI3P.
We used EEA1 as a bona-fide marker of early endosomes vesicles, which are not the only organelles positive for PI3P in the endosomal pathway, as pointed out by the referee. Indeed, while – to our knowledge – there is very little PI3P on recycling endosomes (which are transiently enriched on PI4P en route to plasma membrane (Ketel, K et al, 2016, Nature)), late endosomes and lysosomes could have PI3P positive domains, as seen by colocalization of PI3P with late endosomal markers such as LAMP1 and LAMP2. However, using such markers could be a major issue in our study since these proteins are known to be present on autophagy-related organelles as well, such as autophagolysosomes and autolysosomes. Thus, using late endosome/lysosome markers will not allow to properly discriminate between "endosomal" and "autophagosomal" pools of PI3P. Therefore, we propose to rephrase the description of our experimental framework, highlighting the limitations of our quantitative approach using EEA1 marker in the corresponding paragraph.

2. Suppl.Fig. 9 reports some potentially very important findings, but the data here are not conclusive. Firstly, the images in b are of too low quality to allow meaningful quantifications. Secondly, the counting of LC3-positive structures by itself does not reveal whether autophagic flux is affected. Given the importance of this issue, the authors need to do a proper flux analysis as recommended by Klionsky et al, Autophagy, 2016.

We agree with the referee. Because of the importance of the issue, and in accordance with the editor, we propose to remove this figure and the corresponding results in the manuscript.

Minor point:

The acronyme "PI3KC3" for the class III PI 3-kinase complex is confusing since it strongly resembles "PIK3C3", the gene/protein name for human VPS34.

We apologize for this confusion: we now refer to VPS34 for the protein and to "PI3KC3 complex" for VPS34 and its partners.

2nd Editorial Decision 26 April 2017

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. I appreciate the introduced changes and I am happy to accept your manuscript in principle for publication here.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Etienne Morel
Journal Submitted to: The EMBO Journal
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

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The data shown in figures should satisfy the following conditions:

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

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Is the variance similar between the groups that are being statistically compared?	No estimatation for variance was used.

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