EMBO reports

STK38 kinase acts as XPO1 gatekeeper regulating the nuclear export of autophagy proteins and other cargoes

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Review timeline:

Submission date:	22 March 2019
Editorial Decision:	16 May 2019
Revision received:	9 July 2019
Editorial Decision:	31 July 2019
Revision received:	15 August 2019
Accepted:	3 September 2019

Editor: Martina Rembold

Transaction Report:

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

1st Editorial Decision 16 May 2019

Thank you for the submission of your research manuscript to EMBO reports. I apologize for the delay in handling your manuscript but we have only recently received the last referee report. The full set of reports is copied at the end of this mail.

As you will see, the reviewers acknowledge that the findings are potentially interesting. However, they remain unconvinced that some of the major conclusions are sufficiently supported by the data. In particular, it will be essential to substantiate the data on autophagy and to confirm that the localization of Beclin-1 and YAP depends on XPO1 phosphorylation by STK38 rather than indirect effects mediated via 14-3-3 binding to phosphorylated Beclin-1 or YAP.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

See also our guide for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

3) Supplementary information: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix should include a table of content on the first page and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures according to this nomenclature.

For more details please refer to our guide to authors:
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4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://embor.embopress.org/authorguide#livingorganisms

6) Please also note that we now mandate that all corresponding authors provide an ORCID digital identifier that is linked to his/her EMBO reports account.

7) We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

8) Regarding data quantification and statistics, please ensure to specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable but please note that statistical testing should only be applied if n {greater than or equal to} 3. See:
http://embor.embopress.org/authorguide#statisticalanalysis

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.

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REFEREE REPORTS

Referee #1:

Martin et al. has provided molecular and cellular data for an extensive analysis of how STK38/NDR1 protein kinase and the XPO1 nuclear export protein are functionally related under a nutrient starvation-induced autophage condition. STK38 activates XPO1 by directly phosphorylating XPO1's Ser 1055 residue. Activated XPO1 is required for STK38 nuclear exit and autophage. This work established an interesting mutual activation model involving STK38 and XPO1 proteins. There are a few concerns needed to be addressed:

1) The manuscript appears to focus on autophage to illustrate functional consequences of STK38-XPO1 interaction. Therefore, the importance of the ECM detachment experiment at the beginning of this manuscript is not clear. Maybe this part could be removed or reduced.

2) In addition to directly activating XPO1 in the nucleus, how might STK38 be involved in regulating autophage in cytoplasm (there it is interacting with many proteins). There should be at least some discussion about this aspect as authors have shown that STK38 is critical for inducing autophage.

3). YAP1 has been well established as a direct target of LATS1/LATS2 protein kinases, while STK38 is phosphorylating the same HxRxxS/T consensus sites of YAP1. Some data need to be shown about LATS1/2 expression in A549 cells and whether they are functional in this cell type. This could be a good opportunity for finding out under what conditions STK38 could functionally replace LATS1/2 to phosphorylate YAP1 and promote YAP1 cytoplasmic localization.

4). In references #34 and #36 (may be some other places as well), there are some duplications that have to be removed.

Referee #2:

The study by demonstrates a role for the kinase STK38 in the regulation of XPO1 function via phosphorylation. While the study nicely shows that STK38 phosphorylates XPO1 to modulate cargo export, the experiments related to autophagy are misleading and lacks proper acknowledgements and comparisons with prior work on XPO1 regulation and autophagy. This is particularly problematic since it is one of the key claims of this study and the lack of appropriate citations (easily findable by simply googling xpo1 and autophagy) markedly dampens the enthusiasm. Overall, the work on the STK38/XPO1 connection is interesting, but would undoubtedly gain with appropriate major revisions on the autophagy-related work and on other concerns.

Major comments:

1. Figure 2E is quite misleading as it portrays the autophagic index of KPT-treated and untreated (DMSO) as the same. By normalizing "Complete" as "1.00" across different treatments, the authors give the false impression that autophagy is the same in "Complete" cells treated with XPO1 inhibitors (KPT drugs). Indeed, autophagy has been demonstrated to be increased with KPT-185 and KPT-330 in HeLa cells via TFEB nuclear enrichment (Silvestrini et al. 2018 Cell Reports PMID:29768192). There is a real possibility that starvation cannot further decrease the autophagy index in KPT-treated cells because autophagic flux is already high and can't be further increased upon starvation. Indeed, Figure 2D levels of p62 are slightly decreased in KPT-treated "Complete" cells potentially suggestive of increased autophagy. Therefore, the authors absolutely need to represent the data in Figure 2E so that the autophagy index is comparable across different treatments and the authors must cite this relavent research and comment/perform more experiments to address how XPO1 promotes starvation-induced autophagy in the context of the known cargo protein, TFEB. For e.g.
2. Additionally, the use of delta autophagy index in Figure 2E and elsewhere in the manuscript (Figure 3C) is incompletely showing the data as the initial levels of autophagy in different conditions is not shown and prevent the authors to fully appreciate what the levels of autophagy are in untreated and treated conditions. Thus, it would be ideal to follow the same pattern of graphical representation as in Fig 2, keeping in mind that data needs to be comparable across treatments.

3. It is necessary to show representative images of GFP-LC3-RFP-LC3Δ cells and GFP/RFP puncta being quantified.
4. To complement the use of the GFP/RFP reporter, authors could demonstrate changes in autophagic flux by blotting LC3 and comparing the levels of LC3I and LC3II.
5. On a more manuscript-wide level, it is important to be consistent with the assays used for measuring autophagy instead of reporting only GFP/RFP LC3 ratios in some places and only p62 levels in other places. Please report both in all the panels involving autophagy assessment.
6. In figure 3A, It is odd to observe that Nuc/Cyto ratio for constitutively active STK38 (PIF) is still >1
7. In figure 3C, there is no statistical testing indicated for the reduction in autophagy in PIF mutants compared to WT.
8. There is no quantification of STK38 levels upon starvation (not just redistribution). Supplementary fig S3 has a Western blot in which STK38 levels seem to be reduced upon starvation. There is no insight offered into how increased autophagy upon starvation is accompanied by decreased levels of STK38, Beclin 1 and, to a lesser extent, ATG5.
9. In Figure 4, the authors should determine XPO1 S1055 phosphorylation in presence of constitutively active (PIF) and kinase-dead STK38, as adequate positive and negative controls, respectively.
10. In Figure 5, although it is known that heterozygous mutation C528S of the cargo-binding pocket is sufficient to confer resistance to selinexor (Neggers et al., 2016 Oncotarget), the authors need to specify whether they used a heterozygous mutant or offer an explanation as to how STK38 was exported in the HeLa C528S mutant. Showing STK38 distribution in untreated cells (no KPT) in the C528S mutants is required.
11. In Figure 5D, despite the statistical non-significance, the quantification of p62 levels demonstrates, at best that the loss of XPO1 phosphorylation (S1055A) leads to a partial loss of the autophagic flux. The statement on page 12 that "HPA1 cells carrying a XPO1(S1055A) mutant failed to undergo autophagy when starved of nutrient, as shown by the lack of degradation of the p62 autophagy marker compared to wild-type cells" is an over-interpretation and needs to be rephrased to accurately describe the data (i.e. have reduced autophagy when starved of nutrient, as shown by the limited degradation of the p62 autophagy marker compared to wild-type cells).
12. In Figure 6, the authors fail to mention a previous study that showed that XPO1 inhibition by KPT-185 leads to beclin-1 nuclear accumulation (Zhang K et al. Blood 2014 http://www.bloodjournal.org/content/124/21/3596).
13. In Figure 6C, it is difficult to determine how much the loss of XPO1 phosphorylation (S1055A) affects beclin-1 localization, especially during starvation. A better image and a DAPI staining would help readers appreciate the nuclear/cytoplasm partitioning.
14. In Figures 6 & 7, it is important to use the kinase-dead STK38 mutant to confirm that Beclin 1 and YAP1 localization depends on XPO1 phosphorylation by STK38.
15. In the discussion, the authors do not cite that XPO1 inhibition is known to slow cancer progression by preventing loss of tumor-suppressor proteins (Parikh et al., 2014 J. Hematol. Oncol. PMID:25316614) and do not speculate how their results contribute to the current knowledge of XPO1's involvement in cancer.

Minor comments:

16. The title of the manuscript is not entirely clear, especially the latter half "regulating nuclear export of its functional partners". It is not clear whose functional partners. Also, as the authors themselves state, it remains to be determined if many cargoes follow this mechanism.
17. Table S1 is the same as Fig. 1B, but the text implies that a larger list should be included. I suggest that table S1 shows the entirety of the STK38 interactome dataset.

Referee #3:

Martin et al. describe novel interaction partners of STK38 in the context of starvation-induced autophagy and resistance to anoikis, using proximity-labeling and quantitative SILAC proteomics. These two conditions differentially affect the association of STK38 with cytosolic and nuclear partners. Following up the idea of localization-dependent interactions, the authors show that STK38 shuttles across the nuclear membrane, it interacts with XPO1, and its cytoplasmic relocalization upon starvation depends on this karyopherin. Mechanistically, STK38 is not only a cargo for XPO1,

but it also mediates XPO1 phosphorylation (at residue 1055) and consequent activation. Active XPO1 in turn facilitates the nuclear export of the STK38-binding partner Beclin1 and the STK38-substrate Yap1 as well.

Overall this is a thorough and well-written study, which unravels exciting and novel aspects of XPO1 regulation through STK38, providing new insight of general scientific interest. The experiments are well-controlled, elegant technical approaches and new reagents (e.g. phosphospecific XPO1 antibody) are used, and for the most part the data provide substantial support for the conclusions

Nonetheless, there are some points of criticism as well. These regard the clarity of some findings, occasional over-interpretation of the data, and the requirement of additional proof to make certain aspects of the study more conclusive. It is important to emphasize that these points (below) do not question the overall validity of the work, and should be readily addressable by the authors.

Major points:

The presentation of the MS data is somewhat confusing. The current description suggests that the intersection of the two context-dependent sets (starvation-induced interactors and detachment-induced interactors) contains 50 proteins. Later however it is stated that 19 within these 50 proteins were affected only by detachment but not by starvation. Please explain or correct the description.

Along the same lines, XPO1 had an interaction score of 21 in the resistance to anoikis, but was not found in the autophagy samples, yet the authors use the later conditions throughout the manuscript for studying STK38-XPO1 interactions. Please explain.

The finding that okadaic acid (OA) does not affect the association of STK38 and XPO1 does not justify the conclusion that STK38 phosphorylation (or that of XPO1) is not required for its association with XPO1. This is an over-interpretation of the data because many proteins are phosphorylated on particular residues under basal conditions. This experiment only shows that the OA-induced extra phosphorylation does not alter interaction. The proposed conclusion can be validated only by in vitro phosphatase treatment (depshosphorylation). The author can provide evidence this way or should tone down this conclusion.

XPO1 inhibitors clearly prevent the starvation-induced reduction in nuclear myc-STK38 (Fig 2C) However, the authors indicate a reversal of the effect - in the presence of the inhibitor nutrition deprivation seems to cause significantly higher nuclear levels. The authors show but do not mention and discuss this finding. What could be the explanation? Does this condition unmask an elevated nuclear import as well? Also, it is misleading that that the origin (which is not zero) of the Y axis is not shown. Please use the full scale or indicate the starting value.

While the loss of p62 is indeed grater starved in controls cells (Fig 2D), there is a clear reduction in this protein also in presence of XPO1 inhibitors. Even if the difference, in the current representation (see below) is not significant, it does not seem to be correct to conclude that the inhibitors abolished all effects. To more accurately (and fairly) document these findings, the optimal quantification of the western blots should be considered. In different experiments (several separate blotw) the absolute value of the protein ratios may not be meaningful, and this arbitrariness generates the scatter which masks significant changes. More meaningful quantitation cab be obtained if a) in each experiment the DMSO control is normalized to 1 and the other samples (for that experiment) are expressed accordingly. Thus the control has no scatter or b) in each experiment the complete medium value is 1 and the corresponding value for the starved condition is expressed accordingly. A non-parametric test is to be used then for statistics. Please re-analyze the data accordingly and report if significant changes occur. Similar considerations regard Fig 5D as well.

The cytoplasmic relocalization of Beclin1 and Yap1 upon starvation is convincing and the XPO1 mutants (inactive S1055A and constitutively active S1055D/E) affect their localization as predicted. However, the conclusion that STK38 (generally) regulates the nuclear export of XPO1 cargos has not been sufficiently proven by these examples. Importantly both molecules can associate with or phosphorylated by STK38 directly, which is a complicating factor in interpretation, Specifically,

Beclin1 is a STK38 interaction partner [PMID: 26387716] and it is therefore conceivable that the loss of cytoplasmic STK38 upon STK38 knockdown or upon expression of the XPO1 mutant S1055A account for the observed loss of cytoplasmic Beclin1. Alternatively, Akt activates STK38 [PMID: 22142472] and phosphorylates Beclin1, leading to 14-3-3 interaction [PMID: 23112296]. 14-3-3 interaction is known to increase cytoplasmic localization in many instances. Knockdown of STK38 in cells expressing XPO1 S1055D/E could unambiguously show that Beclin1 shuttling is controlled by (STK38-mediated) XPO1 activation.

Similarly, Yap1 nuclear/cytoplasmic distribution is majorly affected by LATS/NDR phosphorylation and the consequential 14-3-3 binding. Again, lack of STK38 activity (due to knock-down or XPO1 inhibition/mutation) or a potential over-activation by XPO1 S1055E/D might trigger the observed changes in Yap1 localization by affecting Yap1's phosphorylation/14-3-3 binding. Could the authors address these issues by testing Yap1 phosphorylation or testing Yap1 localization upon STK38 knock-down in cells expressing XPO1 S1055E or D?

Alternatively, the impact of XPO1 phosphorylation/mutation on cargo shuttling could be tested by an artificial construct comprising phosphorylation independent NLS and XPO1-specific NES.

Minor comments questions:

Fig S2E: how are the correlations calculated?

Table S1: Please change dimensions so that the full table width fits to one page, and the table headers can be read. What is the average STD for a protein?

Line 106: Please state the minimal fold increase with STD (or the average fold increase) of the 32 interactions

Line 162: Please give a short explanation how the assay works (maybe in the figure legend)
Figure 3C: While this representation focuses on the change in autophagy for each construct, normalized to its own "complete" condition, this depiction somewhat hides the changes induced by these constructs in the absence of starvation (later shown in
Figure 3D). A depiction of Figure 3C&D as in Figure 2E left would be better.

Figure 3D: Please explain the increase in autophagy when expressing inactive STK38

Line 212-213: Please refer to the paper by Kang et al, which describes S1055-doendent interaction of XPO1 with 14-3-3. https://www.tandfonline.com/doi/abs/10.1080/19768354.2013.801366

Line 317: The approach allowed to identify proteins in the vicinity of STK38 (potential interaction partners) and these proteins might or might not be substrates. Please correct your statement.

Line 320-323: The authors have not addressed Yap1 phosphorylation by STK38, therefore they cannot state that the diverse-substrate model of STK38 action was disproved.

1st Revision - authors' response 9 July 2019

Referee #1:

Martin et al. has provided molecular and cellular data for an extensive analysis of how STK38/NDR1 protein kinase and the XPO1 nuclear export protein are functionally related under a nutrient starvation-induced autophage condition. STK38 activates XPO1 by directly phosphorylating XPO1's Ser 1055 residue. Activated XPO1 is required for STK38 nuclear exit and autophage. This work established an interesting mutual activation model involving STK38 and XPO1 proteins. There are a few concerns needed to be addressed:

1) *The manuscript appears to focus on autophage to illustrate functional consequences of STK38-XPO1 interaction. Therefore, the importance of the ECM detachment experiment at the beginning of this manuscript is not clear. Maybe this part could be removed or reduced.*
The reviewer is correct that our manuscript covers different aspects: starting with a comparative study, the manuscript then unfolds with functional data related to only one of the terms of the comparison: autophagy.
Nevertheless, the rationale to zoom on XPO1 as a partner of STK38 arises from the comparison of localization of STK38's partners, suggesting that STK38 may shuttle from the nucleus to the cytoplasm depending on the context. However, we shortened this part in the revised version of the manuscript (lines 112-117).

2) In addition to directly activating XPO1 in the nucleus, how might STK38 be involved in regulating autophage in cytoplasm (there it is interacting with many proteins). There should be at least some discussion about this aspect as authors have shown that STK38 is critical for inducing autophage.
Consistently with the reviewers' request, we enriched the discussion on this topic and we performed an experiment addressing directly the question: are STK38's partners identified by mass spectrometry dealing with autophagy? The question was addressed this way: can the activation of XPO1 by phosphorylation account for the role of STK38 in autophagy? The results are depicted in both Main Figure 5E, and Figure EV3B and recounted at lines 268-278. This experiment demonstrates that once XPO1 is activated (in the present case by the substitution of S1055 by a phosphomimetic residue (S1055D), STK38 seems no more to be required for autophagy as quantified by LC3 dots. The XPO1_S1055D allele is epistatic on a loss of function of STK38 for autophagy. Although there is no known *bona fide* autophagy protein among STK38 partners, we cannot exclude that some of them participate in some fine-tuning of autophagy but at best it is a marginal contribution.
Thus, our claim that the major contribution of STK38 to autophagy is by its activating phosphorylation of XPO1 that allows the cytoplasmic localization of at least Beclin1 and maybe other autophagy actors (ATG1, 3, 4, 10, 12) that were described as XPO1 cargoes (Kirli et al., 2015, eLife, 4:1-28).

3). YAP1 has been well established as a direct target of LATS1/LATS2 protein kinases, while STK38 is phosphorylating the same HxRxxS/T consensus sites of YAP1. Some data need to be shown about LATS1/2 expression in A549 cells and whether they are functional in this cell type. This could be a good opportunity for finding out under what conditions STK38 could functionally replace LATS1/2 to phosphorylate YAP1 and promote YAP1 cytoplasmic localization.
This is an excellent suggestion. We have checked that LATS1/2 promoters are not methylated in A549 (data not shown), as opposed to the promoter of RASSF1 gene. The prediction is that this later methylation leads to a shutdown of the Hippo kinase cascade (including LAST1/2).
We investigated whether indeed YAP1_S127 was phosphorylated and on which kinase this phosphorylation was dependent. YAP1 is indeed phosphorylated on serine S127, but STK38 is not responsible since its effective knock-down by RNAi does not change YAP1_S127 phosphorylation while knock-down of LATS kinases almost annihilated this phosphorylation. This new result supports our model according to which STK38 promotes YAP1 cytoplasmic localization through XPO1 phosphorylation and not YAP1 phosphorylation. These new data and interpretation are reported in the revised manuscript at Appendix Figure S7E&F and lines 321-328.

4). *In references #34 and #36 (may be some other places as well), there are some duplications that have to be removed.*
We removed the duplications in the reference section.

Referee #2:

The study by demonstrates a role for the kinase STK38 in the regulation of XPO1 function via phosphorylation. While the study nicely shows that STK38 phosphorylates XPO1 to modulate cargo export, the experiments related to autophagy are misleading and lacks proper acknowledgements and comparisons with prior work on XPO1 regulation and autophagy. This is particularly problematic since it is one of the key claims of this study and the lack of appropriate citations (easily findable by simply googling xpo1 and autophagy) markedly dampens the enthusiasm.

Overall, the work on the STK38/XPO1 connection is interesting, but would undoubtedly gain with appropriate major revisions on the autophagy-related work and on other concerns.

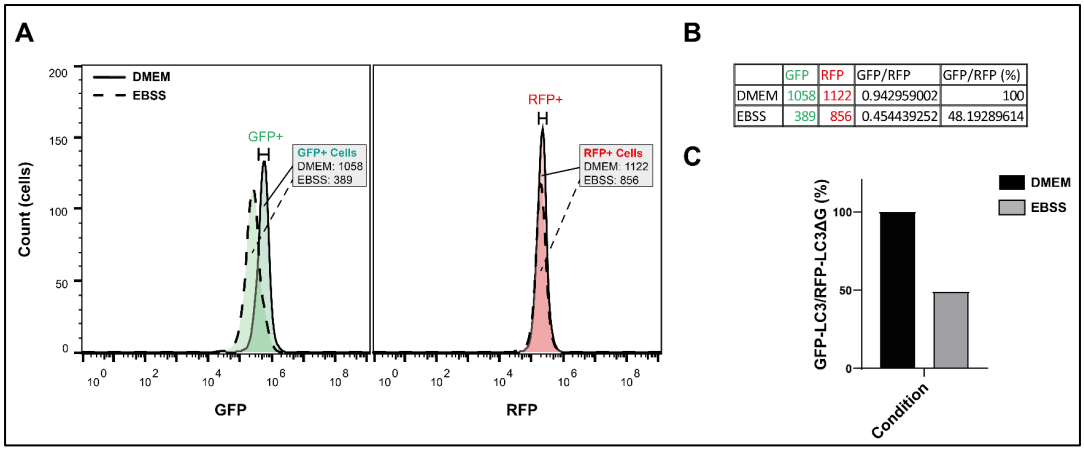
Major comments:

1. Figure 2E is quite misleading as it portrays the autophagic index of KPT-treated and untreated (DMSO) as the same. By normalizing "Complete" as "1.00" across different treatments, the authors give the false impression that autophagy is the same in "Complete" cells treated with XPO1 inhibitors (KPT drugs).
Indeed, autophagy has been demonstrated to be increased with KPT-185 and KPT-330 in HeLa cells via TFEB nuclear enrichment (Silvestrini et al. 2018 Cell Reports PMID:29768192). There is a real possibility that starvation cannot further decrease the autophagy index in KPT-treated cells because autophagic flux is already high and can't be further increased upon starvation.
Indeed, Figure 2D levels of p62 are slightly decreased in KPT-treated "Complete" cells potentially suggestive of increased autophagy. Therefore, the authors absolutely need to represent the data in Figure 2E so that the autophagy index is comparable across different treatments and the authors must cite this relavent research and comment/perform more experiments to address how XPO1 promotes starvation-induced autophagy in the context of the known cargo protein, TFEB. For e.g.
In order to comply with the reviewer's comment, we propose a new representation of data for figures monitoring autophagy with the GFP-LC3-RFP-LC3ΔG probe developed by Kaizuka et al. (Mol. Cell, 2016, 64: 835–849), so that everything is comparable with everything else and was compared accordingly, as indicated by the significance scores (Figures 2E and 3C).
In addition, as requested by reviewers, all autophagy data are now supported by two readouts: p62 degradation and LC3 maturation/degradation (either by FACS (Figures 2E and 3C), Dots (Figures 5E and EV3B), or Western-blotting (Appendix Figure S1A&B).
The reviewer is right, we overlooked the mentioned reference but discussed it now in the revised manuscript (see lines 169-173) and added it in the references section. As explained above, we propose a new representation of data from FACS analysis, where everything is comparable with everything else. Despite the fact that p62 levels are slightly decreased in KPT-treated "Complete" conditions compared to DMSO-treated "Complete" condition, there is no significant difference by comparing the quantifications of these conditions in both p62-based and LC3 (FACS)-based assays.
Our data are in line with Silvestrini et al. (2018). But one should still note that there is a significant difference between their context and ours: KPT concetrations are the same (1 µM) but they probre autophagy after 6 (Lysotracker-based assay)/24 hours (LC3) of treatment, whereas we stopped the experiment at 4 hours. As a global reconciliating conclusion, blocking XPO1 can have two opposite effects : it blocks Beclin1 in the nucleus and thus impairs its availability in the cytoplasm where it is due in order to imitate autophagosome formation / it increases the nuclear concentration of TFEB, leading to overexpression of autophagy genes which leads to some "forced " autophagy. The latter is covered by the Silvestrini publication, the former is ours as presented in the manuscript.

2. Additionally, the use of delta autophagy index in Figure 2E and elsewhere in the manuscript (Figure 3C) is incompletely showing the data as the initial levels of autophagy in different conditions is not shown and prevent the authors to fully appreciate what the levels of autophagy are in untreated and treated conditions. Thus, it would be ideal to follow the same pattern of graphical representation as in Fig 2, keeping in mind that data needs to be comparable across treatments.
As mentioned above in our response to point 1, we propose a new representation of data for these figures (Figures 2E and 3C) in order to comply with reviewer's comment.

3. It is necessary to show representative images of GFP-LC3-RFP-LC3Δ cells and GFP/RFP puncta being quantified.
The GFP-LC3-RFP-LC3Δ probe developed and validated by Kaizuka et al. (Mol. Cell, 2016, 64: 835–849) is a probe intended for autophagy evaluation by FACS, with an advantageous key feature: an internal control, the non-cleaved RFP-LC3Δ, that normalizes for the probe expression level. It is totally different from the "traditional" dual biosensor expressing RFP-GFP-LC3 intended for imaging.
To better explain how the quantifications are made, we included a Figure in our rebuttal (see below). In (**A**) both GFP-LC3 and RFP- LC3Δ signals are recorded by FACS. The gating defining GFP+ and RFP+ populations are performed on the control condition and applied to all other conditions, so the referential is always on the first studied condition (DMSO Complete for Figure 2E and siNT Complete for Figure 3C). Then, in (**B**), the ratio is calculated by dividing the number of GFP+ cells

by the number of RFP+ cells (internal control) and always normalized on the first studied condition. Finally, in (**C**), graphical representation is made based on these quantifications.



	GFP	RFP	GFP/RFP	GFP/RFP (%)
DMEM	1058	1122	0.942959002	100
EBSS	389	856	0.454439252	48.19289614

4. To complement the use of the GFP/RFP reporter, authors could demonstrate changes in autophagic flux by blotting LC3 and comparing the levels of LC3I and LC3II.
We agree with the reviewer and all figures describing autophagy are now depicting two read-outs: p62/SQSTM1 degradation and LC3 monitoring either by FACS analysis or imaging for puncta formation. It is the case all throughout the manuscript.

5. On a more manuscript-wide level, it is important to be consistent with the assays used for measuring autophagy instead of reporting only GFP/RFP LC3 ratios in some places and only p62 levels in other places. Please report both in all the panels involving autophagy assessment.
Reviewer is right and in this revised version of the manuscript, we have complied with this request. Any autophagy claim is now supported by assessing at least two autophagy features.

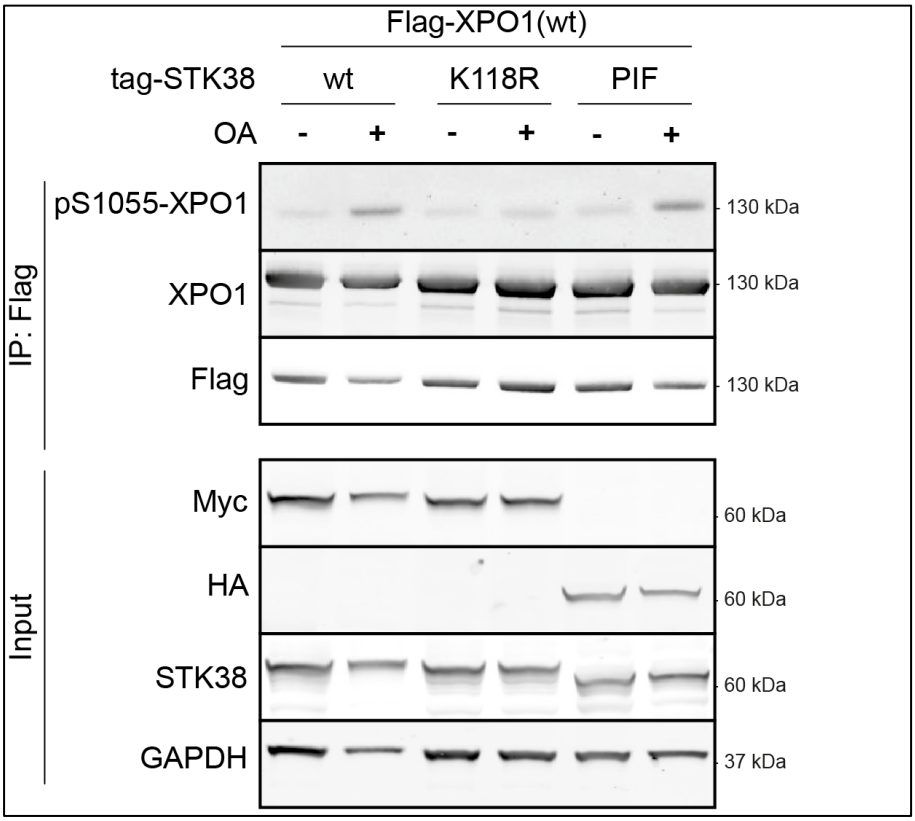
6. In figure 3A, It is odd to observe that Nuc/Cyto ratio for constitutively active STK38 (PIF) is still >1
It is indeed counterintuitive that it remains >1. And the outcome is: we think this ">1" is a mathematical artificial result. The ratio represents not the total amount of signal in each compartment, it represents the total amount of fluorescent signal divided by the surface of interest (mean of fluorescence in Image J). The numerator is the integrated nuclear signal divided by the nuclear surface where the denominator is the cytoplasmic integrated signal divided by the cytoplasm surface. Since cytoplasm surface is bigger than nuclear surface, the denominator gets bigger faster than the nuclear signal decreases. All together at the end the ratio goes over 1.
Example: if we start with an amount given A in the nucleus. Say that upon treatment, half of it goes out then A/2 remains nuclear while A/2 is in cytoplasm. Ratio is 1 but if nuclear A/2 is divided by nuclear surface and cytoplasmic A/2 is divided by cytoplasmic surface, and if cytoplasmic surface is bigger than nuclear surface, then ratio is >1.

7. In figure 3C, there is no statistical testing indicated for the reduction in autophagy in PIF mutants compared to WT.
We fully agree with the reviewer: it is now indicated, and the difference between PIF mutant and WT in complete condition is indeed significant.

8. There is no quantification of STK38 levels upon starvation (not just redistribution). Supplementary fig S3 has a Western blot in which STK38 levels seem to be reduced upon starvation. There is no insight offered into how increased autophagy upon starvation is accompanied by decreased levels of STK38, Beclin 1 and, to a lesser extent, ATG5.
Quantifications were based on means of the four independent experiments and are provided within the figure. Maybe it was not clearly indicated in the legend and we did our best to improve that in the revised version. Even if STK38 seems to be decreased upon starvation the quantifications are within a close range (the differences between image and quantifications are due to the different amount of total protein loaded as can be seen on the GAPDH level). Decrease of proteins (Beclin1

for example) can be due to lysosomal degradation upon starvation because the experiment was performed without the use of any blocker of lysosomal proteolytic activities (chloroquine for example).

9. In Figure 4, the authors should determine XPO1 S1055 phosphorylation in presence of constitutively active (PIF) and kinase-dead STK38, as adequate positive and negative controls, respectively.
This experiment was performed according to the reviewer suggestion and the data are depicted in the figure below.



293T cells were transiently transfected with Flag-XPO1(wt) expression plasmid along with either myc-STK38(wt), myc-STK38(K118R) (kinase-dead variant) or HA-STK38(PIF) (hyperactive mutant) expression plasmids. 24h later, cells were incubated with Okadaic Acid (OA) (final concentration = 1 µM) or DMSO for 1 hour. Flag fusions were immunoprecipited and pulled-down proteins were analyzed by western blotting. Upper panel displays immunoprecipited proteins and lower panel represents whole cell lysates.

For reason beyond our understanding, expression of PIF mutant did not lead to over-phosphorylation of XPO1. Maybe the hyper-activation of WT STK38 upon okadaic acid treatment, which curtails all cellular phosphatase activities, is maximal and cannot by increased anymore. Since these results with the PIF mutant are quite inconclusive, if not even confusing, the experiment was repeated for the sake of reproducibility with WT and kinase-dead mutants. They reproduced well the results shown here above: WT leads to XPO1 Serine1055 phosphorylation upon okadaic acid treatment, and kinase-dead mutant fails to do so. These results are displayed in the revised manuscript as a panel C of Figure 4, which together of panels A&B of this same figure (siSTK38) support the responsibility of the kinase activity of STK38 in this phosphorylation. As a complement on these data in original manuscript, identification of Serine 1055 as the phosphate acceptor was ascertain by mass spectrometry (Appendix Figure S4).

10. In Figure 5, although it is known that heterozygous mutation C528S of the cargo-binding pocket is sufficient to confer resistance to selinexor (Neggers et al., 2016 Oncotarget), the authors need to specify whether they used a heterozygous mutant or offer an explanation as to how STK38 was

exported in the HeLa C528S mutant. Showing STK38 distribution in untreated cells (no KPT) in the C528S mutants is required.
We re-wrote this part (see lines 237-241) for the sake of clarity because homozygote or heterozygote do not apply here. XPO1 mutants are expressed from transiently transfected plasmids while the export of endogenous wt XPO1 was inhibited using KPT-185, so that the only functional XPO1 in the cell are the exogenously mutants.
Previous reports have robustly demonstrated that the C528S mutation does not drastically impair XPO1 export activity as shown by export of cargoes by this mutant and because homozygous mutant cells are viable, the XPO1 export function is essential for cell viablity (Neggers et al., Chem Biol, 2015, 22: 107–116).

11. *In Figure 5D, despite the statistical non-significance, the quantification of p62 levels demonstrates, at best that the loss of XPO1 phosphorylation (S1055A) leads to a partial loss of the autophagic flux. The statement on page 12 that "HPA1 cells carrying a XPO1(S1055A) mutant failed to undergo autophagy when starved of nutrient, as shown by the lack of degradation of the p62 autophagy marker compared to wild-type cells" is an over-interpretation and needs to be rephrased to accurately describe the data (i.e. have reduced autophagy when starved of nutrient, as shown by the limited degradation of the p62 autophagy marker compared to wild-type cells).*
We agree with the reviewer's comment. Although the blot suggests that there is still a degradation of p62 in HAP1_S1055A cells compared to wt cells upon starvation treatment, and that the quantifications (p62/GAPDH) indicate no statistical difference between these conditions, we choose to tone down our conclusions as suggested by the reviewer. Changes have been made in the manuscript (lines 263-265) in agreement with the reviewer's remark.

12. *In Figure 6, the authors fail to mention a previous study that showed that XPO1 inhibition by KPT-185 leads to beclin-1 nuclear accumulation (Zhang K et al. Blood 2014 http://www.bloodjournal.org/content/124/21/3596).*
We added this reference in the manuscript text (line 281) and references section.

13. *In Figure 6C, it is difficult to determine how much the loss of XPO1 phosphorylation (S1055A) affects beclin-1 localization, especially during starvation. A better image and a DAPI staining would help readers appreciate the nuclear/cytoplasm partitioning.*
We added the DAPI staining in the figures displaying endogenous Beclin1 localization in HAP1 cells (now relocated in Figure EV4 B&C), resulting in Beclin1 signal in grey scale and merged pictures with DAPI staining in blue and Beclin1 in yellow.

14. In Figures 6 & 7, it is important to use the kinase-dead STK38 mutant to confirm that Beclin 1 and YAP1 localization depends on XPO1 phosphorylation by STK38.
We used the non-phosphorylable (S1055A) mutant of XPO1 in both Figures (Figure 6B for Beclin1 and Figure 7B for YAP1). In the unfolding of the argument, these figures support the fact that XPO1 phosphorylation is necessary and sufficient to trigger nuclear export (at least for these two tested cargoes). The question whether XPO1 is phosphorylated by STK38 is already supported by other figures and data (Figure 4 for example). Consistently with these later conclusions, we do show that phosphomimetic substitution of XPO1_S1055 makes STK38 unnecessary for XPO1 nuclear export activity, both for Beclin1 (Figure 6C, EV4C, and Appendix Figure S6C) and YAP1 (Figure 7C, EV5C, and Appendix Figure S7D).

15. In the discussion, the authors do not cite that XPO1 inhibition is known to slow cancer progression by preventing loss of tumor-suppressor proteins (Parikh et al., 2014 J. Hematol. Oncol. PMID:25316614) and do not speculate how their results contribute to the current knowledge of XPO1's involvement in cancer.
We added a last paragraph in the discussion (see lines 374-382) as well as the suggested reference.

Minor comments:

16. The title of the manuscript is not entirely clear, especially the latter half "regulating nuclear export of its functional partners". It is not clear whose functional partners. Also, as the authors themselves state, it remains to be determined if many cargoes follow this mechanism.
Indeed, in this manuscript only YAP1 and Beclin1 are demonstrated to be dependent on this mechanism. Although our model provides a framework for the regulation of nuclear export through

relief of the auto-inhibition of XPO1 by phosphorylation by STK38, we are indeed not comprehensive for the more than 4000 predicted cargoes (Kirli et al., 2015, eLife 4:1-28). As mentioned before, we retitled our manuscript "**STK38 kinase, as the XPO1 gatekeeper, regulates nuclear export of autophagy proteins and other cargoes**".

17. *Table S1 is the same as Fig. 1B, but the text implies that a larger list should be included. I suggest that table S1 shows the entirety of the STK38 interactome dataset.*
As the text implies, we do provide the complete list: Table S1 exhibit the entire STK38 interactome identified in this study. The first tab of the Appendix Table S1 displays the whole set of partners identified, the second one displays only the proteins significantly identified in the autophagy context, the third one displays only the proteins significantly identified in the suspension context, and finally, the last tab displays the 50 proteins commonly significantly identified in and common to both contexts.

Referee #3:

Martin et al. describe novel interaction partners of STK38 in the context of starvation-induced autophagy and resistance to anoikis, using proximity-labeling and quantitative SILAC proteomics. These two conditions differentially affect the association of STK38 with cytosolic and nuclear partners. Following up the idea of localization-dependent interactions, the authors show that STK38 shuttles across the nuclear membrane, it interacts with XPO1, and its cytoplasmic relocalization upon starvation depends on this karyopherin. Mechanistically, STK38 is not only a cargo for XPO1, but it also mediates XPO1 phosphorylation (at residue 1055) and consequent activation. Active XPO1 in turn facilitates the nuclear export of the STK38-binding partner Beclin1 and the STK38-substrate Yap1 as well.

Overall this is a thorough and well-written study, which unravels exciting and novel aspects of XPO1 regulation through STK38, providing new insight of general scientific interest. The experiments are well-controlled, elegant technical approaches and new reagents (e.g. phosphospecific XPO1 antibody) are used, and for the most part the data provide substantial support for the conclusions

Nonetheless, there are some points of criticism as well. These regard the clarity of some findings, occasional over-interpretation of the data, and the requirement of additional proof to make certain aspects of the study more conclusive. It is important to emphasize that these points (below) do not question the overall validity of the work, and should be readily addressable by the authors.

Major points:

The presentation of the MS data is somewhat confusing. The current description suggests that the intersection of the two context-dependent sets (starvation-induced interactors and detachment-induced interactors) contains 50 proteins. Later however it is stated that 19 within these 50 proteins were affected only by detachment but not by starvation. Please explain or correct the description.
We agree that this part could be confusing. The text was thoroughly rewritten for the sake of clarity (see lines 112-117). To better explain, 50 proteins were effectively identified in both contexts (starvation-induced autophagy and suspension growth). These 50 proteins were distinguished into two groups according to their association fold with STK38 (non-hierarchical clustering).
The purple cluster is composed of proteins (the 19 above-cited) that mainly increase their interactions (association fold > 1.30) with STK38 when cultured in suspension as compared to cultured in an attached way. On the other hand, these 19 proteins do not see their interaction modified with STK38 upon starvation-induced autophagy as compared to complete medium treatment. We hope this clarifies the data.

Along the same lines, XPO1 had an interaction score of 21 in the resistance to anoikis, but was not found in the autophagy samples, yet the authors use the later conditions throughout the manuscript for studying STK38-XPO1 interactions. Please explain.
The reviewer is correct, we did not find XPO1 in the autophagy samples. To be more precise, looking back to raw mass spectrometry data, only one peptide was found for XPO1 in two of the three replicates of the "starvation" condition, so XPO1 didn't pass the threshold of significance (at

least two unique peptides in all three replicates) and does not appear in the list of "autophagy partners" of STK38. We can speculate why it was significant in one condition and not in the other, but that would be merely inconclusive and a form of hand waving. The reasoning for focusing on XPO1 came from the comparison of the localization of STK38's partners in the different contexts which enabled us to identify XPO1 as a partner of STK38.

The finding that okadaic acid (OA) does not affect the association of STK38 and XPO1 does not justify the conclusion that STK38 phosphorylation (or that of XPO1) is not required for its association with XPO1. This is an over-interpretation of the data because many proteins are phosphorylated on particular residues under basal conditions. This experiment only shows that the OA-induced extra phosphorylation does not alter interaction. The proposed conclusion can be validated only by in vitro phosphatase treatment (depshosphorylation). The author can provide evidence this way or should tone down this conclusion.
We agree and modified our conclusion in agreement with the reviewer's comment by removing the statement that STK38 phosphorylation was not required for its association with XPO1 (see lines 134-136).

XPO1 inhibitors clearly prevent the starvation-induced reduction in nuclear myc-STK38 (Fig 2C) However, the authors indicate a reversal of the effect - in the presence of the inhibitor nutrition deprivation seems to cause significantly higher nuclear levels. The authors show but do not mention and discuss this finding. What could be the explanation? Does this condition unmask an elevated nuclear import as well?
This is an interesting remark but the effect is, although significant, certainly not substantial. Yes, because export is blocked by inhibitors, a light increase of the import activity may be a likely explanation. Whether it is specific or generic remains elusive and we have not solved this conundrum which is beyond the scope of this manuscript, but may be a interesting finding for future research.

Also, it is misleading that that the origin (which is not zero) of the Y axis is not shown. Please use the full scale or indicate the starting value.
We corrected and indicated the starting values of the Y axis for all figures. We choose to start the Y axis at 0.7 for some of them (STK38 nuc/cyto) for a better appreciation of differences between represented conditions.

While the loss of p62 is indeed grater starved in controls cells (Fig 2D), there is a clear reduction in this protein also in presence of XPO1 inhibitors. Even if the difference, in the current representation (see below) is not significant, it does not seem to be correct to conclude that the inhibitors abolished all effects. To more accurately (and fairly) document these findings, the optimal quantification of the western blots should be considered. In different experiments (several separate blotw) the absolute value of the protein ratios may not be meaningful, and this arbitrariness generates the scatter which masks significant changes. More meaningful quantitation cab be obtained if a) in each experiment the DMSO control is normalized to 1 and the other samples (for that experiment) are expressed accordingly. Thus the control has no scatter or b) in each experiment the complete medium value is 1 and the corresponding value for the starved condition is expressed accordingly. A non-parametric test is to be used then for statistics. Please re-analyze the data accordingly and report if significant changes occur. Similar considerations regard Fig 5D as well.
For all western blot quantifications, every condition is normalized on the average of the first studied condition (here, DMSO control). We think that this way of normalizing data is the most robust because it gives scatter on all studied conditions (even the reference one), allowing to evaluate the reproducibility of the experiment along the replicates. Thus, we are in agreement with the referee's first proposition "a) in each experiment the DMSO control is normalized to 1 and the other samples (for that experiment) are expressed accordingly" because all conditions are expressed on normalized DMSO control condition.

The cytoplasmic relocalization of Beclin1 and Yap1 upon starvation is convincing and the XPO1 mutants (inactive S1055A and constitutively active S1055D/E) affect their localization as predicted. However, the conclusion that STK38 (generally) regulates the nuclear export of XPO1 cargos has not been sufficiently proven by these examples. Importantly both molecules can associate with or phosphorylated by STK38 directly, which is a complicating factor in interpretation, Specifically,

Beclin1 is a STK38 interaction partner [PMID: 26387716] and it is therefore conceivable that the loss of cytoplasmic STK38 upon STK38 knockdown or upon expression of the XPO1 mutant S1055A account for the observed loss of cytoplasmic Beclin1. Alternatively, Akt activates STK38 [PMID: 22142472] and phosphorylates Beclin1, leading to 14-3-3 interaction [PMID: 23112296]. 14-3-3 interaction is known to increase cytoplasmic localization in many instances. Knockdown of STK38 in cells expressing XPO1 S1055D/E could unambiguously show that Beclin1 shuttling is controlled by (STK38-mediated) XPO1 activation.
Similarly, Yap1 nuclear/cytoplasmic distribution is majorly affected by LATS/NDR phosphorylation and the consequential 14-3-3 binding. Again, lack of STK38 activity (due to knock-down or XPO1 inhibition/mutation) or a potential over-activation by XPO1 S1055E/D might trigger the observed changes in Yap1 localization by affecting Yap1's phosphorylation/14-3-3 binding. Could the authors address these issues by testing Yap1 phosphorylation or testing Yap1 localization upon STK38 knock-down in cells expressing XPO1 S1055E or D?
Alternatively, the impact of XPO1 phosphorylation/mutation on cargo shuttling could be tested by an artificial construct comprising phosphorylation independent NLS and XPO1-specific NES.
The issue about the relative contribution of the phosphorylation of XPO1 or YAP1 itself by STK38 to their cytoplasmic localization was indeed addressed as follows. It was more thoroughly addressed about YAP1 for the following reasons. If indeed Beclin1 is phosphorylated, it does not harbor any STK38 phosphorylation motif and we never succeeded to show any phosphorylation of Beclin1 by STK38 *in vitro*. As opposed, YAP1 has been shown to be phosphorylated by STK38 on S127 (for which there is a specific phospho antibody) in an intestinal cell line.
We used this latter in A549 cells at high density where YAP1 leaves the nucleus. As expected, YAP1 was phosphorylated on S127. The question whether this phosphorylation depends on LATS1/2 or STK38 was addressed by depleting these proteins by RNAi. The result is unambiguous: phosphorylation of YAP1_S127 is independent of STK38 in this model while it is dependent on LATS1/2. The effect of STK38 on YAP1 nuclear export is not though the phosphorylation of YAP1. These observations are reported in Appendix Figures S7E&F and documented in the manuscript at lines 322-328.
Consistently with this conclusion, phosphomimetic mutants of XPO1 render STK38 superfluous for the export of YAP1 (Figure 7C and Figure EV5C and lines 321-322 in the revised manuscript). This same genetic approach was undertaken for Beclin1: STK38 is unnecessary for Beclin1 cytoplasmic localization XPO1 carries a phosphomimetic mutation (Figure 6C and Figure EV4C and lines 293-295 in the revised manuscript), supporting the model where a phosphorylating activation of XPO1 is the driver of Beclin1 nuclear export.
In both cases, that changes a bit the model where export is driven by a two-shot mechanism:

1. STK38 phosphorylation of XPO1 arms the system, allowing the cargo (YAP1, Beclin1, etc.) to bind on the cargo binding interface of XPO1 for its export.
2. Another phosphorylation creates a 14-3-3 binding site on the cargo (S127 for YAP1 as example), hooking this latter in the cytoplasm.

Minor comments questions:

F*ig S2E: how are the correlations calculated?*
The correlation was calculated using the Pearson correlation coefficient on the association fold of each significantly identified STK38 partner between each replicates of the two studied conditions (corresponding to columns B,C,D,E,F and G in the “All” tab of the Appendix Table S1). The correlation matrix was generated using the corrplot package in R.

Table S1: Please change dimensions so that the full table width fits to one page, and the table headers can be read. What is the average STD for a protein?
We reformatted the table in order to improve readability. The standard deviation of the ratios of the proteins for each condition is now reported next to the fold change for each protein. For significant proteins, the average is 0.18 (for quantification in starvation condition) and 0.11 (for quantification in suspension condition).

Line 106: Please state the minimal fold increase with STD (or the average fold increase) of the 32 interactions

We added the information by indicating the minimal fold increase with STD of the 32 interactions in the Figure 1 legend (see lines 993-994).

Line 162: Please give a short explanation how the assay works (maybe in the figure legend).
We added a short explanation how the assay works in the Figure 2E and Figure 3C legends. We also give a complete explanation in this letter by responding to point 3 of the reviewer #2.

Figure 3C: While this representation focuses on the change in autophagy for each construct, normalized to its own "complete" condition, this depiction somewhat hides the changes induced by these constructs in the absence of starvation (later shown in Figure 3D). A depiction of Figure 3C&D as in Figure 2E left would be better.
As mentioned above, we propose a new representation of data for these figures (Figures 2E and 3C) in order to comply with reviewer's comment.

Figure 3D: Please explain the increase in autophagy when expressing inactive STK38
With the new representation of data for the Figure 3C, there is no significant change in autophagy when expressing inactive STK38 as compared to the expression of wt STK38.

Line 212-213: Please refer to the paper by Kang et al, which describes S1055-doendent interaction of XPO1 with 14-3-3. https://www.tandfonline.com/doi/abs/10.1080/19768354.2013.801366
We now refer to the Kang et al., paper describing that XPO1_S1055 phosphorylation was required for binding with a 14-3-3 in the revised manuscript (line 209) and in the literature reference section, in agreement with the reviewer's comment.

Line 317: The approach allowed to identify proteins in the vicinity of STK38 (potential interaction partners) and these proteins might or might not be substrates. Please correct your statement.
We agree with the reviewer's comment, and modified our statement in the discussion section by changing "substrate" for "partner".

Line 320-323: The authors have not addressed Yap1 phosphorylation by STK38, therefore they cannot state that the diverse-substrate model of STK38 action was disproved.
This issue was addressed as described above in answer to an issue raised by this reviewer and is now documented in Figure 7C and Figure EV5C and in text at lines 322-328.

2nd Editorial Decision 31 July 2019

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 will see, the referees acknowledge that the revised version addressed most of their concerns and all three referees support publication after some remaining issues have been amended. Referee 2 remains concerned about the autophagy part of the study and considers the data presented in agreement with a specific role of XPO1 in starvation-induced autophagy. Referee 3 remains concerned that the representation of p62 protein levels in Figure 2D is not accurate and I agree with this assessment of Figure 2D. The blots clearly show that p62 levels are still lower in the presence of inhibitors and that thus degradation of p62 is not prevented but rather reduced. This is also reflected in the quantification, even though statistical significance might not be reached. Therefore, please address all remaining concerns from referee 2 and 3 in the main manuscript and in a point-by-point response.

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

****

REFEREE REPORTS

Referee #1:

In this revised manuscript, the authors have properly addressed all my concerns.

Referee #2:

The authors have generally answered my concerns, but some key corrections remain. Mainly, the authors need to clearly differentiate between autophagy in fed (complete) state and autophagy induction by starvation since their data cannot be properly interpreted otherwise. They also need to discuss how their nuclear/cytoplasmic data differ from original work on XPO1 targets.

Two major comments:

1. Authors need to improve their explanation (Lines 170-174) on how their data differs from previously published studies on XPO1 inhibition leading to TFEB inclusion in the nucleus and autophagy induction i.e. Silvestrini et al. 2018 Cell Rep, but also following studies by Li et al. 2018 Nat Commun (PMID: 29992949) and Napolitano et al. Nat Commun (PMID: 30120233). These previous studies put XPO1 as a modulator of the export of the autophagy-regulating transcription factor TFEB and therefore inhibiting XPO1 leads to TFEB enrichment in the nucleus and autophagy and lysosome gene induction. TFEB nucleo-cytoplasmic partitioning appears to be quite dynamic and starvation itself (as well as mTOR inhibition) leads to TFEB enrichment in the nucleus. One would predict that XPO1 inhibition would, at best, accelerate such nuclear enrichment of TFEB associated with an enhancement of autophagy in the complete condition. At worst, XPO1 inhibition would not have an additive effect on autophagy induction during starvation.

Instead, the authors find that

a. XPO1 inhibition has no effect on LC3 flux or p62 levels in complete medium after 4 hours (Fig. 2D-E). As authors suggest, if XPO1 inhibition decreased autophagy after 4 hours, p62 levels should be correspondingly increased in the complete medium (Fig. 2D) and the ratio GFP-LC3/RFP-LC3dG should be increased as well. However, this is not what is found in this study. The solution here is to clearly differentiate between basal autophagy and nutrient-induced autophagy. Whether they are mechanistically different (i.e. using similar or different proteins, signaling, etc.) is still unclear. On line 170, please add "XPO1 has to be functional for the resulting starvation-induced autophagy".

b. If indeed STK38 would regulate the export of autophagy proteins relevant to basal autophagy, silencing it should increase p62 levels and the GFP-LC3/RFP-LC3dG ratio. This is not what is found in Figure 3C-D, suggesting that STK38 function is solely relevant for starvation-induced autophagy.

c. When STK38 is constitutively active (PIF), p62 levels in the complete situation are now decreased markedly as is the GFP-LC3/RFP-LC3dG ratio (Fig. 3C-D), which would be suggestive of a role for STK38 beyond starvation-induced autophagy only. The authors present this as evidence of STK38 role in all autophagies, but the bulk of evidence is that STK38 in its native form is specifically involved during starvation only. In addition, there is also the possibility that constitutively active STK38 affects beclin1 activity directly as a binding partner, as highlighted by another reviewer.

Therefore, the authors need to make the distinction that the effect they see for XPO1 inhibition effect on autophagy induction is largely in the context of starvation-induced autophagy. In the rebuttal, the authors mistakenly conclude that their work is in line with Silvestrini et al. 2018 (and other publications aforementioned). However, TFEB is nuclear-localized during starvation and is required to enhance autophagy and lysosomal biogenesis. Perhaps XPO1 inhibition in that specific context becomes inhibitory on autophagy due to STK38 enhanced function. The authors need to elaborate on that aspect because their explanation that the assays were done after 4 hours incubation

instead of 6 hours in Silvestrini et al. 2018 is evasive and misses an opportunity to clearly highlight how their study specifically captures an effect particularly relevant during starvation.

2. Since the nucleo-cytoplasmic partitioning of proteins via XPO1 is dynamic, XPO1 inhibition is predicted to lead to nuclear enrichment of proteins that are actively targeted by XPO1 (Kirli et al. 2015 eLife, PMID: 26673895). However, in Fig. 5A, nucleus/cytoplasm ratio does not change for beclin1 when animals are under XPO1 inhibition and starvation (condition where beclin1 should be more actively exported by XPO1, but is now inhibited by KPT-185 or KPT-330). Therefore, the authors need to highlight that the extent of the effect of KPT-185 and KPT-330 on Beclin1 nucleo-cytoplasmic localization is different from other validated XPO1 targets treated with Leptomycin B (Kirli et al. 2015 eLife, PMID: 26673895).

Referee #3:

This is an elegant MS. The authors has answered the majority of the questions and included meaningful new experimental data according to the suggestions.

I have only one minor comment. I feel that my earlier remark about the quantiation of blots (and specifically that of Fig 2D for p62 rescue) were correct in that the graph indicates non-significant changes when the actual blot shows clear p62 reduction even in the presence of inhibitors. This situation is likely because the scatter of the control eliminates the significance but if each normalized control were compared to its won experimental partners (treated samples on the same blot)a non-parametric test might show significance. I do not request that this be done but I have two suggestions that can clarify this issue;
a) The authors should use the term "reduced" instead of "prevented".
b) Please calculate (using the same graph and representation) significance between column 2 vs. 4 and 2 vs. 6. It is much more meaningful to state that the p62 levels are significantly higher (i.e. partially preserved) in these cases that to indicate ns difference compared to the control.

2nd Revision - authors' response 15 August 2019

Referee #2:

The authors have generally answered my concerns, but some key corrections remain. Mainly, the authors need to clearly differentiate between autophagy in fed (complete) state and autophagy induction by starvation since their data cannot be properly interpreted otherwise. They also need to discuss how their nuclear/cytoplasmic data differ from original work on XPO1 targets.
This point is very acceptable and is even quite welcome; consequently, indeed, we induced this differentiation, implying XPO1 and STK38 in starvation-induced autophagy within the text of the manuscript (see lines 169, 344, 346, and 349).
About “original work on XPO1 targets”, STK38 was found by Kirli et al. (paper already cited in the manuscript) as a cargo carried out of the nucleus by XPO1, but XPO1 was never identified as a substrate of STK38, and even less that this phosphorylation is activating XPO1, that we persuasively show in the present work.

Two major comments:

1. Authors need to improve their explanation (Lines 170-174) on how their data differs from previously published studies on XPO1 inhibition leading to TFEB inclusion in the nucleus and autophagy induction i.e. Silvestrini et al. 2018 Cell Rep, but also following studies by Li et al. 2018 Nat Commun (PMID: 29992949) and Napolitano et al. Nat Commun (PMID: 30120233). These previous studies put XPO1 as a modulator of the export of the autophagy-regulating transcription factor TFEB and therefore inhibiting XPO1 leads to TFEB enrichment in the nucleus and autophagy and lysosome gene induction. TFEB nucleo-cytoplasmic partitioning appears to be quite dynamic and starvation itself (as well as mTOR inhibition) leads to TFEB enrichment in the nucleus. One would predict that XPO1 inhibition would, at best, accelerate such nuclear enrichment of TFEB

associated with an enhancement of autophagy in the complete condition. At worst, XPO1 inhibition would not have an additive effect on autophagy induction during starvation.

Instead, the authors find that

a. XPO1 inhibition has no effect on LC3 flux or p62 levels in complete medium after 4 hours (Fig. 2D-E). As authors suggest, if XPO1 inhibition decreased autophagy after 4 hours, p62 levels should be correspondingly increased in the complete medium (Fig. 2D) and the ratio GFP-LC3/RFP-LC3dG should be increased as well. However, this is not what is found in this study. The solution here is to clearly differentiate between basal autophagy and nutrient-induced autophagy. Whether they are mechanistically different (i.e. using similar or different proteins, signaling, etc.) is still unclear. On line 170, please add "XPO1 has to be functional for the resulting starvation-induced autophagy".
We thank the reviewer for the suggestion and the sentence was indeed added (see line 169), since our genetic or pharmacological manipulation of the XPO1-STK38 couple impact starvation-induced autophagy more than basal autophagy.

b. If indeed STK38 would regulate the export of autophagy proteins relevant to basal autophagy, silencing it should increase p62 levels and the GFP-LC3/RFP-LC3dG ratio. This is not what is found in Figure 3C-D, suggesting that STK38 function is solely relevant for starvation-induced autophagy.
We thank the referee for his/her comment. In fact, it seems from the result shown in Figure 3 and from our previous work (Joffre et al., Current Biology, 2015, 25:2479) that STK38 function is not relevant in basal autophagy.

c. When STK38 is constitutively active (PIF), p62 levels in the complete situation are now decreased markedly as is the GFP-LC3/RFP-LC3dG ratio (Fig. 3C-D), which would be suggestive of a role for STK38 beyond starvation-induced autophagy only. The authors present this as evidence of STK38 role in all autophagies, but the bulk of evidence is that STK38 in its native form is specifically involved during starvation only. In addition, there is also the possibility that constitutively active STK38 affects beclin1 activity directly as a binding partner, as highlighted by another reviewer.
The reviewer is right. There are at least two possible outcome:

1. As suggested by reviewer, that what is observed is unrelated to the STK38-XPO1 couple (but PIF mutant induces strong auto-export from nucleus, arguing for a triggering of the nuclear cytoplasmic shuttling in Fig3A) and related to other aspects of STK38 biology as its association with Beclin1. We cannot rule out this possibility.
2. As suggested by our data (Joffre et al., Current Biology, 2015, 25:2479) with the fly homologue of STK38, activation of STK38 is instructive for autophagy.

Therefore, the authors need to make the distinction that the effect they see for XPO1 inhibition effect on autophagy induction is largely in the context of starvation-induced autophagy. In the rebuttal, the authors mistakenly conclude that their work is in line with Silvestrini et al. 2018 (and other publications aforementioned). However, TFEB is nuclear-localized during starvation and is required to enhance autophagy and lysosomal biogenesis. Perhaps XPO1 inhibition in that specific context becomes inhibitory on autophagy due to STK38 enhanced function. The authors need to elaborate on that aspect because their explanation that the assays were done after 4 hours incubation instead of 6 hours in Silvestrini et al. 2018 is evasive and misses an opportunity to clearly highlight how their study specifically captures an effect particularly relevant during starvation.
We largely amended the text to incorporate this thoughtful remark (see lines 173-175). Agreeing with the reviewer, we now point to the fact that besides the kinetic difference (6h versus 4h), which might be anecdotic or not, TFEB affects basal autophagy while the XPO1-STK38 duo impacts starvation-induced autophagy.

2. Since the nucleo-cytoplasmic partitioning of proteins via XPO1 is dynamic, XPO1 inhibition is predicted to lead to nuclear enrichment of proteins that are actively targeted by XPO1 (Kirli et al. 2015 eLife, PMID: 26673895). However, in Fig. 5A, nucleus/cytoplasm ratio does not change for beclin1 when animals are under XPO1 inhibition and starvation (condition where beclin1 should be more actively exported by XPO1, but is now inhibited by KPT-185 or KPT-330). Therefore, the

authors need to highlight that the extent of the effect of KPT-185 and KPT-330 on Beclin1 nucleo-cytoplasmic localization is different from other validated XPO1 targets treated with Leptomycin B (Kirli et al. 2015 eLife, PMID: 26673895).
I think that the reviewer is refereeing to Figure 6A instead of Figure 5A. In addition, no animals were used, only cell lines as indicated all along the manuscript and on the Figures themselves. I don't agree with the reviewer's comment about the effect of XPO1 inhibition on Beclin1 behaviour. These results are very consistent with the ones reported by the group of Beth Levine (Liang et al., Cancer Res, 2001, PMID: 11309306 (already cited in the manuscript)) showing that Beclin1 is retained in the nucleus under Leptomycin B treatment (thus the similarity is more qualitatively supported than quantitatively).

Referee #3:

This is an elegant MS. The authors has answered the majority of the questions and included meaningful new experimental data according to the suggestions.
We are very grateful to reviewer for this positive statement.

I have only one minor comment. I feel that my earlier remark about the quantiation of blots (and specifically that of Fig 2D for p62 rescue) were correct in that the graph indicates non-significant changes when the actual blot shows clear p62 reduction even in the presence of inhibitors. This situation is likely because the scatter of the control eliminates the significance but if each normalized control were compared to its won experimental partners (treated samples on the same blot)a non-parametric test might show significance. I do not request that this be done but I have two suggestions that can clarify this issue;
a) The authors should use the term "reduced" instead of "prevented".
We complied and replaced the term "prevented" by "reduced" in the present revised manuscript (see line 156, in red).

b) Please calculate (using the same graph and representation) significance between column 2 vs. 4 and 2 vs. 6. It is much more meaningful to state that the p62 levels are significantly higher (i.e. partially preserved) in these cases that to indicate ns difference compared to the control.
We thank the referee for his/her valuable comment on the analysis of the p62 levels. Accordingly, we have calculated the significance, using a non-parametric test, between column 2 vs. 4 and 2 vs. 6 after normalizing each experimental partner with its own experimental control, as requested.
We point to the fact that starvation-induced autophagy is (significantly) reduced. However, reduced is not annihilated, so reviewer is right to claim that according to p62 amounts, some autophagy is preserved.
This remark is in line with suggestion of reviewer2 and the sentence now is (page 8, lines 154-156): As expected, p62 levels decreased upon starvation (Figure 2D). However, the inhibition of XPO1 with the subsequent inhibition of STK38 cytoplasmic localization reduced p62 degradation (Figure 2D),

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Corresponding Author Name: Jacques Camonis
Manusript Number: EMBOR-2019-48150

Reporting Checklist For Life Sciences Articles

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 (see link list at top right).

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 only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- 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 (see link list at top right).

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were performed in independant triplicate. At least 30 objects (cells) were analyzed from these independant triplicate for IF experiments. See figure legends
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 were excluded for 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 pre-alocated with no post-processing allocation.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Samples were pre-alocated with no post-processing allocation.
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: Statistical significance was quantified by p-values using Graphpad Prism v5.0 software. Student's t test was used if data followed normal distribution
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal (Gaussian) distribution was assessed using both Anderson-darling test, Shapiro-Wilk normality test, and d'Agostino-Pearson normality test (GraphPad Prism V5). See page 34
Is there an estimate of variation within each group of data?	Variation between each group of data was not specifically assessed. Only statistical tests indicated in the figure legend were performed.
Is the variance similar between the groups that are being statistically compared?	No, the variance between groups was not specifically assessed, see above.

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).	Catalog number and Suppplier are always referenced in the manuscript (material and methods section, page 19).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines have been authentified by STR profiling and were routinely ensured to be mycoplasma free.

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D- Animal Models

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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E- Human Subjects

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F- Data Accessibility

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