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FKBP8 recruits LC3A to mediate Parkin-independent mitophagy

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Transaction Report:

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

1st Editorial Decision 05 September 2016

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

As you will see, while the referees acknowledge that the findings are potentially interesting, they all point out that significant revisions are required and that the data need to be strengthened before the study can be considered for publication here. The referees raise a number of - often overlapping - concerns regarding the co-localization data, statistical quantification and the use of overexpressed proteins. From the analysis of these comments it becomes clear that significant revision is required to strengthen the data and conclusions before the manuscript becomes suitable for publication in EMBO reports. However, given the constructive comments and the potential interest of your findings, I would like to give you the opportunity to address these concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board.

Basically, all points raised by the referees need to be addressed. In particular statistical quantification has to be added for Western blot and imaging data, and the interaction should be verified with endogenous proteins. All requested controls have to be provided. Should you decide to embark on such a revision, 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.

REFEREE REPORTS

Referee #1:

The manuscript by Zambarlal et al., makes the very interesting discovery that FKBP8 can function as a mitophagy receptor via LIR mediated interactions with Atg8 proteins. In cells FKBP8 shows preferential binding to a specific Atg8 family member, LC3A. Overall the study will be of high interest to the mitophagy and general autophagy field. The biochemical analyses of FKBP8 including identification of the LIR sequence and Atg8 binding experiments are of very high quality. The cell biology of FKBP8 in mitophagy and its function in recruiting LC3A in cells as determined by the authors is not in doubt, however some experiments (primarily the co-localization studies) lack the scientific rigour expected for EMBO Reports. For example, much of the co-localization and LC3A recruitment analyses rely on one experiment, often conducted in one cell, using a single line of analysis that only represents a fraction of the cell volume to show co-localization. The manuscript would therefore benefit greatly with a more thorough analysis including analyses of multiple cells across more than one experiment. Detailed suggestions as well as other comments are included below.

Specific Comments:

1) Fig. 2A-D The co-localization studies in this figure would benefit greatly by using Pearson's correlation coefficient (or similar) of multiple cells. As it is, analysis of a single line, within a single cell, in one experiment is not entirely sufficient. It should be noted that this reviewer does not doubt the authors conclusion that FKBP8 recruits LC3A, just that the manuscript would benefit from a more thorough analysis.

2) Fig 2B: Is the level of co-localization of FKBP8, LC3A and Tom20 higher than non CCCP treated cells? Quantitation by Pearson's correlation coefficient (or similar) of multiple cells in more than one experiment (as suggested above), would help confirm whether CCCP induces greater co-localization as suggested by the authors.

3) Fig 2E. The western blot is low quality relative to the nice co-IP data in Fig 2G. Furthermore, little to no FKBP8 was co-precipitated in the +CCCP conditions. It appears that there is less FKBP8 after CCCP treatment. Is FKBP8 turned over after mitochondrial depolarization?

4) Fig 2F. Is the expression level of LC3A lower in FKBP8 KO cells? It is difficult to be sure because the blot is overexposed. Can a lower exposure be included or a total fraction to determine whether there are equal levels of LC3A transfected in WT and FKBP8 KO cells? This can affect the interpretation of the amount of LC3A recruited. The more telling comparison in this figure is the -/+ CCCP difference in which there is little to no change in the FKBP8 KO cells.

Fig 2G. This interesting result suggests that additional mitochondrial factors are involved in FKBP8-LC3A binding/recruitment. The authors may want to mention this in the discussion.

Fig 3C. What is the intensity value relative to? Untransfected cells? What is used as the 100% baseline?

Fig 3D and E. As mentioned in earlier comments, Pearson's correlation coefficient would be more informative in these co-localization analyses.

EV6. It is difficult to determine whether LC3A was not highly co-imunopreciptated with NIX and BNIP3 in the absence of a co-IP of FKBP8 as a comparative control.

Fig 4B. The authors propose that BNIP3 and NIX are less efficient at mitophagy however are they expressed at the same level as FKBP8? A western blot showing equal expression levels would support the conclusion made by the authors.

Fig 4. The data in this figure show that simultaneous overexpression of both FKBP8 and LC3A are required to induce mitophagy. It is unclear why overexpression of LC3A is also required and why endogenous LC3A is not sufficient. Can the authors provide a rationale for this result?

Minor comment: Cerulean has been misspelled in the manuscript.

Referee #2:

This is an interesting report by Zambarlal and colleagues who identified FKBP8 as a novel interactor of LC3B via a yeast two-hybrid screen and mapped the domain of interaction to a canonical LIR motif residing in the N-terminus of FKBP8. Importantly, they elucidated a novel function for the mitochondrial-localized FKBP8 as a mitophagy receptor that is important for the recruitment of LC3 to damaged mitochondria in a parkin-independent manner. Consistent with other reports, they showed that FKBP8 escaped degradation during the mitophagy process. Overall, these findings are novel and help contribute to the growing literature on parkin-independent mitophagy. Notwithstanding this, I have several comments on the manuscript in its present form, as discussed below:

1. The interaction studies were largely based on the overexpression of either FKBP8 or LC3. It would be good to show that the endogenous proteins interact with each other.

2. Whilst the lack of recruitment of LC3A by FKBP8 LIR mutant is anticipated, it is curious to note that overexpression of FKBP8 LIR mutant triggers the aggregation of LC3A into prominent puncta, both in control and CCCP-treated cells.

3. Figs. 2E & F need quantification.

4. Fig. 2F: Can the reduction in LC3A level in FKB8-KO cells be rescued by the genetic reintroduction of wild type FKB8 (e.g. via ectopic expression)?

5. Fig. 2G: If wild type FKBP8 is largely localized to the mitochondria, why does it not also recruit more membrane-bound LC3A to the mitochondria (i.e. in a manner similar to its FKBP-Mito counterpart)?

6. Fig. 3: Difficult to interpret whether reduction in TOM20 signal in FKB8 overexpressing cells is due to fragmentation or mitophagy. Related to this, if it is the former, is FKBP overxpression promoting mitochondrial fragmentation through the upregulation of pro-fission proteins or downregulation of pro-fusion proteins?

7. Fig. 3B & C and Fig. 4B: Include statistical evaluation.

8. Fig.5: Do the authors observe the localization of FKBP8 to the ER under these conditions where FKBP8 is thought to escape from degrading mitochondria? Related to this, it would be interesting to include the mitochondrial-localized FKBP mutant in these experiments to examine if it gets acidified along with the mitochondria it decorates (since it presumably cannot escape in this case).

9. Although Parkin may not be involved in FKBP8-mediated mitophagy, is PINK1 accumulation on the OMM a pre-requisite?

10. Given that FKBP8 can recruit LC3A to the mitochondria in the absence of CCCP treatment/mitochondria deplorization, would mitophagy not be taking place constitutively? What is the physiological relevance of such a partnership between FKBP8 and LC3A?

Minor:

- Fig 2E: Which of the two bands is endogenous FKBP8?

- Fig. 2G and EV3C: Do the numbers below the blot refers to the level of LC3-I or II or both? For EV3, puzzling why GFP lane (0.74) is higher than GFP-FKBP8-DTM lane (0.48) when the signal intensity of LC3-I and II is clearly indicating otherwise.

Referee #3:

This manuscript by Zambarlal et al. suggests a possible role for FKBP8 in Parkin-independent

mitophagy. The paper is well written and the authors convincingly demonstrate that FKBP8 interacts with LC3A via its LIR motif in vitro and in over-expression in vivo models. However the evidence for an endogenous interaction between the two proteins and for a role for FKBP8 in mitophagy is, in my opinion, far less convincing. Moreover statistical quantifications are consistently lacking throughout the manuscript, leading the authors to make a number of overstatements/speculations/approximations.

Specific comments:

- While the experiments in Figure 1 appear to be well controlled, the authors failed to convince me that endogenous FKBP8 interacts with FLAG-LC3A at the mitochondria (Figure 2E). (1) The Western blot is messy (yet the FKBP8 antibody seems to be quite specific according to Figure 2F), and a number of controls are missing (E.g IP with control Ig, no LC3 over-expression, IP in FKBP8-KO cell line). (2) The experiment should be repeated in the cytosolic fraction as a control. (3) How do the authors explain that the LC3A levels are not increased in the mitochondrial input from cells treated with CCCP as compared to DMSO?
- Figures 2 A-D should be repeated with endogenous FKBP8 and endogenous LC3 (or WIPI2) staining, to rule out experimental artefacts due to overexpression and/or formation of large GFP-LC3 aggregates (Mizushima et al, Cell 2010). Is LC3A recruited to mitochondria after CCCP treatment in FKBP8-KO line used in Figure 2F?
- In Figure 2F, the authors should quantify LC3-I and II ratio before they draw any conclusion. The blots for the cytosolic fraction are over-exposed.
- In Figure 2G, the authors claim that "FKBP8-Mito precipitated significantly more of the lipidated form of LC3A than WT, ER-bound or cytoplasmic FKBP8". How can they compare FKBP8-Mito and -ER when the two samples have been run in two separate experiments (Figure EV3C) and LC3II/I ratios have been calculated out of n=1 experiment?
- All experiments should be quantified. No (or close to none) mention of statistics is made throughout the entire manuscript.
- In Figure 3, the authors should use a negative control (E.g a GFP-mitochondrial protein which overexpression doesn't lead to a reduction of the TOM20 staining), in order to rule out markers cross-contamination. The authors claim that "significant morphological changes of mitochondria were observed, including fragmentation, dotted structures and perinuclear aggregates". How significant? How was that measured?
- Figure 4 is interesting. Have the authors tried to over-express FKBP8-ER and - TM constructs?
- A vast majority of the experiments are performed in FKBP8 over-expressing cells, they should be repeated in the FKBP8-KO cell line (whenever possible).

1st Revision - authors' response 31 January 2017

We are grateful for the opportunity to submit a revised version of our manuscript. The revision process has led to one additional main figure. Hence, we suggest as, opened for by the Editor, that the revised manuscript is regarded as a Research Article.
In the revised manuscript we have addressed all the points raised by the referees. Quantifications (Figs 2-6 and EV2 And EV4) and statistics (Fig. 3, 4 and 6 and EV2) have been included in all main figures. The interaction between FKBP8 and LC3A has been verified for endogenous proteins (Fig 2A and B), including the requested IgG control.

In the revised manuscript only Fig 1 and Fig 5 (formerly Fig. 4) are kept unchanged from the original manuscript. During the revision period we have conducted a series of new experiments and repeated those at least three times.

We thank all the three reviewers for their detailed reading of our manuscript and their insightful comments, which clearly has helped us to improve our paper. Below we have addressed point-by-point all the comments made by the reviewers.

Referee #1:

The manuscript by Zambarlal et al., makes the very interesting discovery that FKBP8 can function as a mitophagy receptor via LIR mediated interactions with Atg8 proteins. In cells FKBP8 shows

preferential binding to a specific Atg8 family member, LC3A. Overall the study will be of high interest to the mitophagy and general autophagy field. The biochemical analyses of FKBP8 including identification of the LIR sequence and Atg8 binding experiments are of very high quality. The cell biology of FKBP8 in mitophagy and its function in recruiting LC3A in cells as determined by the authors is not in doubt, however some experiments (primarily the co-localization studies) lack the scientific rigour expected for EMBO Reports. For example, much of the co-localization and LC3A recruitment analyses rely on one experiment, often conducted in one cell, using a single line of analysis that only represents a fraction of the cell volume to show co-localization. The manuscript would therefore benefit greatly with a more thorough analysis including analyses of multiple cells across more than one experiment. Detailed suggestions as well as other comments are included below.

Answers to the comments made by reviewer #1:

We are very happy that reviewer #1 finds our discovery ìthat FKBP8 can function as a mitophagy receptor via LIR mediated interactions with Atg8 proteins very interesting and of high interest to the mitophagy and general autophagy field! In the revised version we have thoroughly addressed the question about scientific rigour and performed a number of new experiments that have been quantified to substantiate the observations made.

Specific Comments:

1) Fig. 2A-D The co-localization studies in this figure would benefit greatly by using Pearson's correlation coefficient (or similar) of multiple cells. As it is, analysis of a single line, within a single cell, in one experiment is not entirely sufficient. It should be noted that this reviewer does not doubt the authors conclusion that FKBP8 recruits LC3A, just that the manuscript would benefit from a more thorough analysis.

Answer: We thank the reviewer for pointing this out and completely agree. Figure 2 A-D is Figure 3 in the revised MS, with graphs representing quantitations of the co-localization between mitochondria and FKBP8 in Figure 3B, or LC3A and FKBP8 in Figure 3C. The quantitations were performed by manual analysis of 20-30 cells for each condition in three independent experiments. Furthermore, images of cells expressing FKBP8-Mito (Fig 3) or FKBP8-ER (Fig EV3B) together with LC3A have been included as additional controls together with Vector Control together with LC3A (Fig EV3A). These three controls clearly show that FKBP8 has the ability to recruit LC3A to subcellular structures upon CCCP treatment. We found that quantitations using Pearsons¥s corr. coeff. (or Manders) were not well suited in these experiments because the proteins studied are located both on cellular structures and in specific dots and many structures partially overlap when visualized by confocal fluorescence microscopy.

2) Fig 2B: Is the level of co-localization of FKBP8, LC3A and Tom20 higher than non CCCP treated cells? Quantitation by Pearson's correlation coefficient (or similar) of multiple cells in more than one experiment (as suggested above), would help confirm whether CCCP induces greater co-localization as suggested by the authors.

Answer: Fig 2B is part of Figure 3A in the revised MS. Localization of FKBP8 on mitochondria (identified by the mCherry-OMP25TM probe in the revised MS) is independent on CCCP. Actually, CCCP treatment induces reduced localization of FKBP8 on mitochondria ñ as shown by the graphs in Fig 3B. This is in line with other studies, showing mitochondrial escape of FKBP8 upon CCCP treatment. However, CCCP strongly enhances the FKBP8-LC3A co-localization as presented quantitatively in Fig 3C.

3) Fig 2E. The western blot is low quality relative to the nice co-IP data in Fig 2G. Furthermore, little to no FKBP8 was co-precipitated in the +CCCP conditions. It appears that there is less FKBP8 after CCCP treatment. Is FKBP8 turned over after mitochondrial depolarization?

Answer: We agree that the WB is of low quality, and we have performed new co-IPs presented in Figure 2A and B in the revised MS. The IgG control is included. We used SH-SY5Y cells since this cell line express relative higher amounts of both FKBP8 and LC3A than HeLa (shown if Fig EV2A).

Fig 2A displays nice co-precipitation also after CCCP treatment. FKBP8 is not turned over after mitochondrial depolarization. As mentioned, it escapes from the mitochondria as described under 2) and thereby avoids degradation.

4) Fig 2F. Is the expression level of LC3A lower in FKBP8 KO cells? It is difficult to be sure because the blot is overexposed. Can a lower exposure be included or a total fraction to determine whether there are equal levels of LC3A transfected in WT and FKBP8 KO cells? This can affect the interpretation of the amount of LC3A recruited. The more telling comparison in this figure is the -/+ CCCP difference in which there is little to no change in the FKBP8 KO cells.

Answer: The data presented in the original Figure 2F has been replaced with new data from both HeLa FKBP8 knock-out (Fig 2E in revised MS), and HEK293 knock-out clones (Fig EV2F in revised MS). Furthermore, in the revised MS we have extended the experiment by including blots of extracts from KO cells with reintroduced FKBP8 (Fig 2E and EV2F). In figure 2F the LC3A-II versus LC3A-I ratio have been calculated, and the graphs show the average of three independent experiments with standard deviation. (Variation of transfection efficiency of LC3A between WT and the different clones will not affect the LC3A II/I ratio within each lane). The quantifications presented in Fig 2F show that reintroduction of FKBP8 in the KO clones significantly enhances the amount of lipidated LC3A in the mitochondrial fraction.

Fig 2G. This interesting result suggests that additional mitochondrial factors are involved in FKBP8-LC3A binding/recruitment. The authors may want to mention this in the discussion.

Answer: We agree, the more efficient co-IP of lipidated LC3A to FKBP8 Mito compared to WT FKBP8, suggests that additional mitochondrial factors may enhance the association of FKBP8 and LC3A.

Fig 3C. What is the intensity value relative to? Untransfected cells? What is used as the 100% baseline?

Answer: Thank you for the comment. Unfortunately, there was a misleading labelling of the Y-axis. It should not say Relative. In the revised MS Fig 4B represent Fig 3 B and C. Here we have used cells transfected with Myc-OMP25TM expression plasmid as negative control, and set the TOM20 intensity and area in these cells as 100%. Furthermore, student T-test is included to verify the significance of the intensity and area changes.

Fig 3D and E. As mentioned in earlier comments, Pearson's correlation coefficient would be more informative in these co-localization analyses.

Answer: We agree, but since Pearsonís correlation coefficient was found not to be applicable on localization patterns including both dots and subcellular structures with partial overlaps (see point 1. above) we have substituted the images in Fig 3D/E with the co-immunoprecipitation assay shown in Fig 4C. Fig 4C shows that Flag-LC3A precipitates substantial amounts of FKBP8 from a HeLa cell extract, but hardly any BNIP3 or NIX. This shows that LC3A binds more strongly to FKBP8 than to the mitophagy receptors BNIP3 and NIX.

EV6. It is difficult to determine whether LC3A was not highly co-imunopreciptated with NIX and BNIP3 in the absence of a co-IP of FKBP8 as a comparative control.

Answer: The experiment was repeated 3 times with FKBP8 in parallel, and one representative result is shown in the revised MS Figure 4C.

Fig 4B. The authors propose that BNIP3 and NIX are less efficient at mitophagy however are they expressed at the same level as FKBP8? A western blot showing equal expression levels would support the conclusion made by the authors.

Answer: A western blot showing the expression levels of the various mitophagy receptor constructs are included in Fig EV4C in the revised manuscript.

Fig 4. The data in this figure show that simultaneous overexpression of both FKBP8 and LC3A are

required to induce mitophagy. It is unclear why overexpression of LC3A is also required and why endogenous LC3A is not sufficient. Can the authors provide a rationale for this result?

Answer: As Figure 4D shows, in our assays all the mitophagy receptors (BNIP3, NIX and FKBP8) tested required overexpression of their preferred ATG8 homologue for detectable acidification of mitochondria. This way the need for a damaging stimulus to the mitochondria is alleviated. We do not find this to be unexpected, but it is interesting that both the ATG8s and the mitophagy receptors need to be co-overexpressed in the absence of a stress stimulus. To induce a significant level of mitophagy without applying a stress stimulus overexpression is needed.

Minor comment: Cerulean has been misspelled in the manuscript.

Answer: Thank you for spotting the misspelling. The spelling is corrected in the revised MS.

Referee #2:

This is an interesting report by Zambarlal and colleagues who identified FKBP8 as a novel interactor of LC3B via a yeast two-hybrid screen and mapped the domain of interaction to a canonical LIR motif residing in the N-terminus of FKBP8. Importantly, they elucidated a novel function for the mitochondrial-localized FKBP8 as a mitophagy receptor that is important for the recruitment of LC3 to damaged mitochondria in a parkin-independent manner. Consistent with other reports, they showed that FKBP8escaped degradation during the mitophagy process. Overall, these findings are novel and help contribute to the growing literature on parkin-independent mitophagy. Notwithstanding this, I have several comments on the manuscript in its present form, as discussed below:

1. The interaction studies were largely based on the overexpression of either FKBP8 or LC3. It would be good to show that the endogenous proteins interact with each other.

Answer: We are happy that the reviewer finds the study interesting and important! We have performed new Co-IP experiments of endogenous FKBP8 and LC3A proteins from SH-SY5Y cells, and included proper controls. The result shown in the revised manuscript Figure 2A and B verify interaction between the endogenous proteins.

2. Whilst the lack of recruitment of LC3A by FKBP8 LIR mutant is anticipated, it is curious to note that overexpression of FKBP8 LIR mutant triggers the aggregation of LC3A into prominent puncta, both in control and CCCP-treated cells.

Answer: The puncta formed by YFP-LC3A over-expression is not dependent on the FKBP8 LIR mutant, as YFP-LC3A forms similar puncta when it is over-expressed together with empty vector control (Fig EV3A). Hence, FKBP8-LIRm has no impact on LC3A localization neither under normal conditions nor under CCCP treatment.

3. Figs. 2E & F need quantification.

Answer: In the revised MS, new data (see new Fig 2) have replaced the original Figs 2E and F and the new data have been quantitated where relevant.

4. Fig. 2F: Can the reduction in LC3A level in FKB8-KO cells be rescued by the genetic reintroduction of wild type FKB8 (e.g. via ectopic expression)?

Answer: In the revised manuscript, the experiment presented in Fig 2F (Fig 2E and EV2F in revised MS) has been repeated, but with additional lanes including FKBP8-KO cell lines with reintroduced FKBP8. The results presented in the revised Fig 2E and F and Fig. 2EVF and G, clearly show that FKBP8 facilitates recruitment of lipidated LC3A to mitochondria.

5. Fig. 2G: If wild type FKBP8 is largely localized to the mitochondria, why does it not also recruit more membrane-bound LC3A to the mitochondria (i.e. in a manner similar to its FKBP-Mito counterpart)?

Answer: WT FKBP8 on mitochondria is dynamic and escapes to the ER upon mitophagy induced by co-overexpression with LC3A. Hence, the net result is that WT FKBP8 may not recruit LC3A to the mitochondria as efficiently as the FKBP8-Mito construct. Importantly, WT FKBP8 recruits slightly more LC3A than the FKBP8-ER and deltaTM constructs.

6. Fig. 3: Difficult to interpret whether reduction in TOM20 signal in FKB8 overexpressing cells is due to fragmentation or mitophagy. Related to this, if it is the former, is FKBP overxpression promoting mitochondrial fragmentation through the upregulation of pro-fission proteins or downregulation of pro-fusion proteins?

Answer: Thank you for valuable comments. However, we believe that the data represent fragmentation since LC3A is not co-expressed with FKBP8. In Fig 5B we show that the mitophagy receptors, including FKBP8, is dependent on over-expression of their corresponding ATG8 protein to induce mitophagy under normal conditions. We have performed western blotting to measure the expression of mitofusion 2 upon FKBP8 overexpression. No change in mitofusion expression could be detected (data not shown).

7. Fig. 3B & C and Fig. 4B: Include statistical evaluation.

Answer: Statistics are included in the revised manuscript, showing that the reduction in intensity and area of TOM20 staining is significant.

8. Fig.5: Do the authors observe the localization of FKBP8 to the ER under these conditions where FKBP8 is thought to escape from degrading mitochondria? Related to this, it would be interesting to include the mitochondrial-localized FKBP mutant in these experiments to examine if it gets acidified along with the mitochondria it decorates (since it presumably cannot escape in this case).

Answer: Thank you for this valuable comment. In the revised MS, Fig 6 represents an extended version of the previous Fig 5. As the referee suggested, we have include the FKBP8 mutant FKBP8-Mito, and in the Fig EV5B, also the FKBP8-N412K mutant that is shown to be unable to undergo mitochondrial escape. The acidified mitochondria that contains colocalised FKBP8 WT, -Mito or -N412K are quantitated in two independent experiments and represented by graphs in Fig 6B. Nearly 100% of the acidified mitochondria contain colocalised FKBP8-Mito of FKBP8-N412K. In contrast, in cells expressing FKBP8-WT only 40% of the acidified mitochondria contained FKBP8. Instead, FKBP8-WT was enriched on subcellular structures/dots outside the mitochondria. This indicates that FKBP8-WT escapes to ER (as shown previously) during mitophagy.

9. Although Parkin may not be involved in FKBP8-mediated mitophagy, is PINK1 accumulation on the OMM a pre-requisite?

Answer: Unfortunately, we were unable to find a PINK1 antibody that worked in IF so that we could investigate PINK1 accumulation on the mitochondria. Western blots of mitochondrial fractions displayed no appearant change in PINK1 upon FKBP8 overexpression.

10. Given that FKBP8 can recruit LC3A to the mitochondria in the absence of CCCP treatment/mitochondria deplorization, would mitophagy not be taking place constitutively? What is the physiological relevance of such a partnership between FKBP8 and LC3A?

Answer: In the absence of CCCP, LC3A needs to be over-expressed together with FKBP8 to induce mitophagy. Hence, enrichment of LC3A – or increased affinity in the FKBP8-LC3A binding in one or another way seems to be necessary to facilitate FKBP8 driven mitophagy. Depolarized mitochondria may induce a local enrichment of LC3A.

Minor:

- Fig 2E: Which of the two bands is endogenous FKBP8?

Answer: The blot represented immunoprecipitated endogenous FKBP8 (M 50-60 kDa). The upper band is FKBP8, while the lower band was the IgG (MW approx. 55 kDa). In the revised MS, the Co-IP is repeated with the IgG control included, and shown in Fig 2A and B.

- Fig. 2G and EV3C: Do the numbers below the blot refers to the level of LC3-I or II or both? For EV3, puzzling why GFP lane (0.74) is higher than GFP-FKBP8-DTM lane (0.48) when the signal intensity of LC3-I and II is clearly indicating otherwise.

Answer: The numbers below represent ed the LC3I/LC3-II ration. This was indicated to the right in the Figure. In the revised MS, Fig 2D represent the previous Fig 2G. The numbers still represent the LC3A II/ LC3A I ratio, and this is indicated to the right of the figure and in the figure legend.

Referee #3:

This manuscript by Zambarlal et al. suggests a possible role for FKBP8 in Parkin-independent mitophagy. The paper is well written and the authors convincingly demonstrate that FKBP8 interacts with LC3A via its LIR motif in vitro and in over-expression in vivo models. However the evidence for an endogenous interaction between the two proteins and for a role for FKBP8 in mitophagy is, in my opinion, far less convincing. Moreover statistical quantifications are consistently lacking throughout the manuscript, leading the authors to make a number of overstatements/speculations/approximations.

Specific comments:

- While the experiments in Figure 1 appear to be well controlled, the authors failed to convince me that endogenous FKBP8 interacts with FLAG-LC3A at the mitochondria (Figure 2E). (1) The Western blot is messy (yet the FKBP8 antibody seems to be quite specific according to Figure 2F), and a number of controls are missing (E.g IP with control Ig, no LC3 over-expression, IP in FKBP8-KO cell line). (2) The experiment should be repeated in the cytosolic fraction as a control. (3) How do the authors explain that the LC3A levels are not increased in the mitochondrial input from cells treated with CCCP as compared to DMSO?

Answer: (1) We agree that the Western blot in Figure 2E was of low quality. The endogenous IP has been repeated in SH-SY5Y cells including IgG controls, and shown in Fig 2A and B in the revised MS. (2) The cytosolic fraction did not contain detectable amounts of FKBP8, hence IP of FKBP8 from this fraction will not give any bands on the gel. (3) According to Fig 2E and F and Fig EV2F and G, in the revised MS, the LC3A - and especially the LC3A II form, increases in the mitochondrial fraction upon CCCP treatment.

- Figures 2 A-D should be repeated with endogenous FKBP8 and endogenous LC3 (or WIPI2) staining, to rule out experimental artefacts due to overexpression and/or formation of large GFP-LC3 aggregates (Mizushima et al, Cell 2010). Is LC3A recruited to mitochondria after CCCP treatment in FKBP8-KO line used in Figure 2F?

Answer: Unfortunately, we have not found any antibody for LC3A and FKBP8 that works well in IF, hence repeating 2A-D with the endogenous proteins can not be done. However, we have selected cells with relatively low expression levels of FKBP8 and LC3A for quantitation. Furthermore, we have include the construct FKBP8-Mito and FKBP8-ER as controls, in addition to the vector control. These results, shown in Fig 3A-C and Ev3, clearly show that mitochondrial FKBP8 with an intact LIR motif, has the ability to recruit LC3A to the mitochondria ñ and this recruitment is significantly enhanced upon CCCP treatment. FKBP8-ER recruits LC3A to the ER upon CCCP treatment, while FKBP8-LIRm do not co-localize with LC3A upon CCCP treatment.

Yes, LC3A is recruited to mitochondria after CCCP treatment in FKBP8-KO cells, as shown in Fig 2E and Fig EV2F, indicating that LC3A can also be recruited in a FKBP8 independent manner. However, the recruitment is significantly enhanced when FKBP8 is reconstituted in the KO cells.

- In Figure 2F, the authors should quantify LC3-I and II ratio before they draw any conclusion. The blots for the cytosolic fraction are over-exposed.

Answer: The experiments presented in Figure 2F, presented in Fig 2E and F and Fig EV2F and G, in the revised MS, are repeated including FKBP8-KO cell lines rescued with FKBP8 over-expression. These revised Figs clearly show that FKBP8 facilitates mitochondrial recruitment of the

LC3A II form.

- In Figure 2G, the authors claim that "FKBP8-Mito precipitated significantly more of the lipidated form of LC3A than WT, ER-bound or cytoplasmic FKBP8". How can they compare FKBP8-Mito and -ER when the two samples have been run in two separate experiments (Figure EV3C) and LC3II/I ratios have been calculated out of n=1 experiment?

Answer: The experiment has been repeated in the revised manuscript, and the samples are run on the same gel (revised Figure 2D). Similar results were obtained in three independent experiments.

- All experiments should be quantified. No (or close to none) mention of statistics is made throughout the entire manuscript.

Answer: Quantifications (Figs 2-6 and EV2 And EV4) and statistics (Fig. 3, 4 and 6 and EV2) have been included in all main figures.

- In Figure 3, the authors should use a negative control (E.g a GFP-mitochondrial protein which overexpression doesn't lead to a reduction of the TOM20 staining), in order to rule out markers cross-contamination. The authors claim that "significant morphological changes of mitochondria were observed, including fragmentation, dotted structures and perinuclear aggregates". How significant? How was that measured?

Answer: In the revised MS the result previously presented in Fig 3 is presented in Fig 4. The mitochondrial marker protein Myc-OMP25TM has been included as the negative control. The fragmentation was measured as loss of Myc-OMP25TM intensity and area using the Volocity quantification program. The average values with standard deviations from three independent experiments are shown in Fig 4B.

- Figure 4 is interesting. Have the authors tried to over-express FKBP8-ER and - ΔTM constructs?

Answer: Yes, in the revised manuscript Figure EV4 we show results for the FKBP8-ER and -deltaTM constructs. The data clearly show that FKBP8 has to be associated with the mitochondria to efficiently induce mitophagy together with LC3A.

- A vast majority of the experiments are performed in FKBP8 over-expressing cells, they should be repeated in the FKBP8-KO cell line (whenever possible).

Answer: Unfortunately, the established FKBP8 KO cell lines undergo apoptosis upon CCCP treatment. Hence, they are very sensitive to CCCP and generally sensitive to apoptotic stimuli. This is in line with previous studies showing that FKBP8 is an anti-apoptotic protein. We have applied the KO cell lines in Fig 2. But because of the sensitivity of the KO cell lines, we instead have chosen to study WT cells displaying very modest ectopic expression of FKBP8 and LC3A in the imaging analysis. This is now mentioned in the Results section of the revised MS.

We look forward to your response and hope that our manuscript can be found acceptable for publication in EMBO Reports.

2nd Editorial Decision 28 February 2017

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for my delayed response but we have only now received the last referee report and all reports are copied below.

As you will see, all referees are very positive about the study and request only some minor revisions and clarifications. While the analysis of endogenous LC3 is certainly appreciated (reviewer 1, point 1), it is not required for publication here, as you have already performed a major round of revision and I think that the analysis of endogenous LC3 in all experiments would extend the scope of a second revision.

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

- You have submitted your manuscript as Scientific Report. As the number of figures and the length of the manuscript exceeds the limit for reports (5 figures, 25,000 ± 2,000 characters) I suggest to resubmit the manuscript as Article. In this case the Results and Discussion section can stay as it is now, i.e., as separate sections.

- The Expanded View content is limited to 5 items. Please submit Table EV1 as Appendix. The Appendix is a single pdf file that includes a table of content on the first page, and the respective tables. Please reformat the references of the Appendix according to the numbered style of EMBO reports.

- Statistics and data quantification: the information is currently incomplete or unclear in the legends of Fig. EV1A and Fig. 4B. Moreover, the legend of Fig. 6B also states 'n=100'. I assume that 100 cells were counted. Could you please indicate the number of replicates? Please note that in case 100 cells were counted from one replicate (n=1) the calculation of p-values is not appropriate. Along these lines, referee 2 indicated missing statistics for Fig. 2F, but as the number of replicates does not exceed 2, the calculation of p-values would also not be appropriate in this case.

- Please provide scale bars for the panels in EV3A, EV3C (ROI) and EV4A (ROI).

- I noticed a typo in the legend for Fig. 2E,F: "... the graph in D represents the average values..." should refer to F.

- Please remove the statement that refers to the Expanded View Information that you have currently inserted before the Acknowledgements.

- We routinely perform a quality check of all submitted figure files and images. This scan triggered an inconsistent background modification in the Figures 4A, EV1E, EV3C, EV4A. The cutoff for the lower intensity values (background) of the different images appears to have been set differently. This is in principle fine but could you please for clarification specify if the different images were modified differently and submit the source data for these images?

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Overall the authors have addressed the comments and concerns resulting in a greatly improved manuscript. However, a couple of minor points remain which the author's may wish to address.

1) Reviewer 3 has raised valid concerns regarding LC3A and FKBP8 over expression and suggested analysing endogenous proteins. The authors responded that they were unable to find suitable antibodies for IF of the endogenous proteins. Cell signaling has an LC3A antibody (#4599) that detects endogenous LC3A in IF that they might want to try out.

2) Do the authors have a loading control available for Fig EV2A?

3) In Fig 4 C please indicate which band is Nix and which one is BNIP3 as there are numerous bands and it is currently unclear.

4) Figure 4B: The figure legend indicates N=40 suggesting 40 experiments but it is more likely that 40 cells were counted. Were 40 cells counted per independent experiment with an N=3? Please clarify.

5) The authors state in the discussion that this study is the first demonstration of LC3A in mitophagy. However, while the manuscript was in review, a study was published showing LC3A

can participate in Parkin mediated mitophagy (Nguyen et al 2016 J Cell Biol).

Referee #2:

Overall, the authors have revised the manuscript to my satisfaction. I am happy to see that they have carried out new experiments in response to my queries. Just two minor comments:

1. Fig 2F: Provide statistics

2. Fig. 2E-F: Although overexpression of FKBP8 as anticipated enhances the LC3A levels in the mitochondrial fraction, the reduction in LC3A recruitment to the mitochondria previously observed in FKBPKO cells is curiously not so evident here.

Referee #3:

The authors have addressed most of my concerns and I believe the manuscript is now suitable for publication in EMBO reports.

2nd Revision - authors' response 07 March 2017

We are delighted to learn that our paper now can be accepted provided following minor revisions and clarifications. Below we have first answered or addressed the points made by the editor and thereafter answered the points made by the reviewers.

Answers to the points made by the Editor:

- You have submitted your manuscript as Scientific Report. As the number of figures and the length of the manuscript exceeds the limit for reports (5 figures, 25,000 {plus minus} 2,000 characters) I suggest to resubmit the manuscript as Article. In this case the Results and Discussion section can stay as it is now, i.e., as separate sections.

Answer: We now resubmit the manuscript as Article. As agreed with the editor we technically submit as a Scientific Report and then the editor or editorial assistant will change manuscript type to Article in the submission system.

- The Expanded View content is limited to 5 items. Please submit Table EV1 as Appendix. The Appendix is a single pdf file that includes a table of content on the first page, and the respective tables. Please reformat the references of the Appendix according to the numbered style of EMBO reports.

Answer: We now have placed the previous Table EV1 as Appendix and reformatted the reference to the to the numbered style of EMBO reports.

- Statistics and data quantification: the information is currently incomplete or unclear in the legends of Fig. EV1A and Fig. 4B. Moreover, the legend of Fig. 6B also states 'n=100'. I assume that 100 cells were counted. Could you please indicate the number of replicates? Please note that in case 100 cells were counted from one replicate (n=1) the calculation of p-values is not appropriate. Along these lines, referee 2 indicated missing statistics for Fig. 2F, but as the number of replicates does not exceed 2, the calculation of p-values would also not be appropriate in this case.

Answer: For Fig. EV1A we have corrected the legend as follows: "The bar graphs in the top panel represent the mean values of percentage binding of in vitro translated FKBP8 to GST-LC3A or -LC3B WT and mutant proteins relative to the 10% input with standard deviations based on three independent experiments."

For Fig. 4B, we have removed the stars and indications of p-values in the figure and wrote the following in the figure legend to clarify: "The bars represents the average values with standard deviations of TOM20 positive area and intensity per cell based on analyzing 40 cells for each protein. The experiment was performed twice with similar results."

For Fig. 6B, we also removed the stars and indications of p-values in the figure and clarified the text in the figure legend accordingly: "The bars represent the average values from at least 100 cells in two independent experiments. Error bars represent s.d." As the editor mentions, calculations of p-values would also not be appropriate for Fig. 2F.

- Please provide scale bars for the panels in EV3A, EV3C (ROI) and EV4A (ROI).

Answer: This is done in the final version.

- I noticed a typo in the legend for Fig. 2E,F: "... the graph in D represents the average values..." should refer to F.

Answer: This is corrected in the final version.

- Please remove the statement that refers to the Expanded View Information that you have currently inserted before the Acknowledgements.

Answer: This is done in the final version.

- We routinely perform a quality check of all submitted figure files and images. This scan triggered an inconsistent background modification in the Figures 4A, EV1E, EV3C, EV4A. The cutoff for the lower intensity values (background) of the different images appears to have been set differently. This is in principle fine but could you please for clarification specify if the different images were modified differently and submit the source data for these images?

Answer: The images may have been adjusted a bit in the final software we prepare the figures in to aid visualization. We have now submitted the source data for the figures in question.

Answers to the comments made by reviewers:

We thank all the three reviewers for their detailed reading of our manuscript, insightful comments and constructive criticisms. This has been of great help in the revision process and has clearly aided us in improving our paper. Below we have addressed point-by-point the comments made by the reviewers to our revised version.

Referee #1:

Overall the authors have addressed the comments and concerns resulting in a greatly improved manuscript. However, a couple of minor points remain which the author's may wish to address.

1) Reviewer 3 has raised valid concerns regarding LC3A and FKBP8 over expression and suggested analysing endogenous proteins. The authors responded that they were unable to find suitable antibodies for IF of the endogenous proteins. Cell signaling has an LC3A antibody (#4599) that detects endogenous LC3A in IF that they might want to try out.

Answer: We thank the reviewer for suggesting this antibody.

2) Do the authors have a loading control available for Fig EV2A?

Answer: In the final revised version we have replaced this figure with a new one including loading control.

3) In Fig 4 C please indicate which band is Nix and which one is BNIP3 as there are numerous bands and it is currently unclear.

Answer: This is now indicated with asterisks and explained in the figure legend.

4) Figure 4B: The figure legend indicates N=40 suggesting 40 experiments but it is more likely that

40 cells were counted. Were 40 cells counted per independent experiment with an N=3? Please clarify.

Answer: The reviewer is correct in assuming that 40 cells were counted per independent experiment. This is now clarified in the figure legend.

5) The authors state in the discussion that this study is the first demonstration of LC3A in mitophagy. However, while the manuscript was in review, a study was published showing LC3A can participate in Parkin mediated mitophagy (Nguyen et al 2016 J Cell Biol).

Answer: The study of Nguyen et al 2016 J Cell Biol show that when all ATG8 family are knocked out by CRISPR/CAS9 technology and single ATG8 family are re-expressed one by one in the KO cell line LC3A is able to mediate Parkin-dependent mitophagy and starvation-dependent autophagy. This is not the same as we show where LC3A and FKBP8 together have a particular role in Parkin-independent mitophagy. To acknowledge the findings by Nguyen et al (which support a role for LC3A in mitophagy) we have referred to this paper and modified our text in the Discussion like this: "… and this is the first study indicating a particular role for LC3A in mitophagy. Consistently, a very recent study by Ngyuen et al. showed that in a background where all ATG8 members were knocked out, re-expression of LC3A could mediate Parkin-dependent mitophagy [34]."

Referee #2:

Overall, the authors have revised the manuscript to my satisfaction. I am happy to see that they have carried out new experiments in response to my queries. Just two minor comments:

1. Fig 2F: Provide statistics

Answer: As pointed out by the Editor: "Along these lines, referee 2 indicated missing statistics for Fig. 2F, but as the number of replicates does not exceed 2, the calculation of p-values would also not be appropriate in this case."

2. Fig. 2E-F: Although overexpression of FKBP8 as anticipated enhances the LC3A levels in the mitochondrial fraction, the reduction in LC3A recruitment to the mitochondria previously observed in FKBPKO cells is curiously not so evident here.

Answer: The original version had blots from HEK293 FKBP8 KO cells. We have now replaced these with blots from HeLa FKBP8 KO cells because almost all of our experiments were performed in HeLa cells (which do not express Parkin), making this KO line most relevant. We also show HEK293 FKBP8 KO cell blots in EV2F (quantified LC3A II/I in EV2G). The HEK293 FKBP8 KO clone is also a different clone from the one used in the original version of the paper. This latter clone showed more variation in LC3A recruitment between experiments whereas the results obtained with the clone shown in Fig. EV2F-G were very reproducible. We therefore chose this clone for the final experiments during the revision of the paper.

We look forward to your response and hope that our manuscript now is acceptable for publication in EMBO Reports.

3rd Editorial Decision 10 March 2017

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Terje Johansen
Journal Submitted to: EMBO Report
Manuscript Number:

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.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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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http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Every experiment has been repeated independently at least 3 times to ensure reproducibility. For quantitation and localisation studies performed by imaging we determined the amount of cells to be analyzed prior the experimental upset. The number of cells selected were based on experience from our previous work on how many cells are needed for generating statistical relevant data.
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?	In EV2F,G the experiments were repeated 5 times or more, and the lowest and highest values were excluded from calculation of average and s.d. These criteria were pre-established.
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.	The following experiments and analysis were performed by at least two persons, to avoid subjective bias. Fig. 2, Fig 3, Fig. 4, Fig. 6, EV2, EV3, EV4, EV5
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.	Confocal imaging, quantitations and colocalisation studies presented in Figs 3, 4, 6, EVEV3 and EV5 were performed by at least two different persons. Western blots presented in Figs 2C, 2D,2E, 2F, 4C and EV2F , EV2G were performed of at least two persons.
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?	Paired student T-Test is used as statistical test in Figs 3C, 4B, 6B, EV2G.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes, standard deviations are included in Figs 1D, 2E, 3B, 3C, 4B, 5B, 6B, EV2G, EV4B.
Is the variance similar between the groups that are being statistically compared?	Yes, the variance is similar between groups.

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).	The LC3A and LC3A/B antibodies are commercial. Their catalog number is stated in Material and Methods section, and we have validated them by WB on Cell-extracts over-expressing LC3A or LC3B. The FKBP8 Ab is commercial and was verified by WB on the HeLa and Hek FKBP8 KO cell lines (see EV2). The GFP, Myc, Flag antibodies, and the scondary antibodies, are all commercial, well established antibodies. Their catalogue numbers are in Material and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We regularly test our cell lines for mycoplasma, and all cell lines used was found to be mycoplasma negative. The source of the cell lines used is stated in the Material and Methods section.

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

D- Animal Models

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No