EMBO reports

UBXD8 mediates mitochondria-associated degradation to restrain apoptosis and mitophagy

Jing Zheng, Yu Cao, Jun Yang and Hui Jiang
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Dear Dr. Jiang

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also raise a number of largely overlapping concerns and have a number of suggestions for how the study should be strengthened, including the need for stronger, biochemical evidence that UBXD8 localizes to mitochondria. I think that all points raised by the referees are important and should be addressed.

In addition to the concerns raised by the referees, I would like to share a comment I had received from the expert advisor I had discussed your manuscript with before having it peer reviewed. The advisor commented that you exclude a role for UBXD8 in degrading mitochondrial preproteins but do not provide evidence whether the analysed precursor proteins associate with TOM complexes. While it is not mandatory to address this comment experimentally, you might want to consider it to further strengthen your study.

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.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (June 18, 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

*** IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.***

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

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5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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

8) Figure legends and data quantification:
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat
- Please also include scale bars in all microscopy images.

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

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this manuscript, Zheng et al. report the role of UBXD8, an adaptor of the mammalian AAA-ATPase VCP, in mitochondria-associated degradation (MAD) of proteins acting in apoptosis and mitochondria-specific autophagy (mitophagy). VCP is an evolutionarily conserved multi-tasking enzymes critical for unfolding and extracting proteins from large complexes and organelles. In yeast, the VCP homolog Cdc48 interacts with Ubx2, an adaptor that is anchored to mitochondria and the endoplasmic reticulum (ER), promoting MAD and ER-associated degradation (ERAD), respectively. Whether VCP also mediates MAD in mammals remains unknown. Through a proteomics approach for ubiquitinated mitochondrial proteins and their interacting partners, the authors identified UBXD8 that has been suggested to act in VCP-mediated ERAD and lipid droplet-associated degradation. They performed a series of immunofluorescence imaging and cell fractionation, and found that UBXD8 also localizes to mitochondria and facilitates recruitment of VCP to mitochondria. In addition, mitochondria- and ER-resident ubiquitin E3 ligases co-immunoprecipitated with UBXD8. Cells lacking UBXD8 accumulated the mitochondrial proteins MiD49 and Mcl-1, and the ER protein Insig1. Interestingly, exclusively mitochondria-anchored UBXD8 variant (mito-UBXD8) restored efficient degradation of Mcl-1 and Insig1, indicating that the MAD system promotes degradation of substrates on both mitochondria and the ER. Additional results further suggest that UBXD8 acts in protection of cells against inducers of mitochondrial stress and apoptosis, and suppression of mitophagy and apoptosis via degradation of Bnip3 and Noxa, respectively.

The data in this study are well-organized and could provide new insights into the molecular mechanisms of MAD involving VCP and UBXD8 in mammals. However, it remains unclear if ER-localized VCP and UBXD8 promotes both ERAD in cis and MAD in trans. In addition, whether MAD requires UBXD8-dependent recruitment of VCP to mitochondria has not been investigated. In conclusion, this study would significantly be strengthened if the authors clarify these issues and address the following points.

Specific points:

1. In Figure 1F, the authors should perform a subcellular fractionation assay to examine fractions of whole cell homogenate, cytosol, mitochondria, and the ER by immunoblotting with antibodies specific for UBXD8, VCP, and representative markers. Note that the assay requires density gradient ultracentrifugation to separate mitochondria from the ER.

2. In Figure 2B, the authors should repeat the immunoprecipitation assays to clearly detect UBXD8 and VCP co-precipitated with 3HA-MARCH5 and 3HA-MUL1. It would also be better to test if the endogenous UBXD8 can be co-immunoprecipitated with the endogenous VCP, MARCH5, and RNF185.

3. Exclusively ER-anchored UBXD8 (ER-UBXD8) and mito-UBXD8 should be expressed in cells lacking the endogenous UBXD8 and examined as described in Figures 2G-H, 4A, 4C, and 5B-D.

4. The authors should quantify the protein levels analyzed in Figures 2C-E, 2H, 4A, and 4C.

5. UBX*, a UBXD8 mutant defective in VCP binding (Figure S2A), should be expressed in cells lacking the endogenous UBXD8 and examined as described in Figures 1F-G, 2H, 4A, 4C, and 5B-D. It would also be great to generate mito-UBX* and ER-UBX*, and analyze them as described in Figures 2G-H, 4A, 4C, and 5B-D.

6. In Figure 5B-C, the authors should investigate autophagy-defective mutants lacking UBXD8 and demonstrate that an increase in mitophagy-positive signals by loss of UBXD8 indeed depends on autophagy.

Referee #2:

In the present manuscript Zheng et al. investigate a putative mitochondrial function of the protein UBXD8, previously described to function at the endoplasmic reticulum and lipid droplets. The authors localize a fraction of UBXD8 to mitochondria, and present data pointing to a UBXD8 dependent recruitment of VCP to mitochondria. They identify several mitochondrial substrates of UBXD8 and thereby link the function of UBXD8 to apoptosis and mitophagy. The manuscript provides potentially very interesting insights into the turnover of proteins at the mitochondrial outer membrane. However, additional data are required to

show the specificity of the findings.

Major points:

1. The localization of UBXD8 is of central importance for the conclusions of this manuscript. The authors used fluorescence microscopy to determine the cellular localization of UBXD8. The authors should also utilize an alternative methods like cellular fractionation to show the localization of endogenous UBXD8. The authors may also show the mass spectrometry data of mitochondrial proteins to reveal how they identified UBXD8 in a mitochondrial fraction.

2. In Figure 1F the authors present isolated mitochondria, in which VCP is reduced in absence of UBXD8 suggesting that UBXD8 recruits VCP to mitochondria. Given the normally high abundance of ER in a mitochondrial preparation, the authors should show an ER protein as control.

3. Are the identified mitochondrial UBXD8 substrates involved in mitophagy and apoptosis degraded in a VCP dependent manner? Does the VCP-binding deficient variant UBXD8 UBX* as presented in Figure S2A show increased sensitivity to apoptosis and increased autophagy rates?

4. The authors report that mitochondrial UBXD8 mediates the turnover of the ER protein Insig1-FLAG, while ER resident UBXD2 also mediates turnover of the mitochondrial protein Mid49. To exclude mislocalization of the substrate proteins, it would be important to assess the subcellular localization of Insig1-FLAG and Mid49 in UBXD8 and UBXD2 deficient cells.

5. The authors observe increased sensitivity of cells lacking UBXD8 to actinomycin D and doxorubicin, which they link to increased apoptosis. The authors should provide additional data to support this conclusion like cytochrome c release. Furthermore, it is not entirely clear whether Bnip3 is degraded in a UBXD8-dependent manner. The immunostainings in Figure 4 for Bnip3 and Nix mark several bands. It remains unknown which is the specific signal.

Minor points:

Line 305: The authors propose that UBXD8 is an essential MAD component. This needs to be rephrased as UBXD8 is not essential for cell survival. The loss of UBXD8 does not abrogate degradation of the identified substrates, indicating further quality control pathways. Thus, UBXD8 is not an essential MAD component.

The authors state that the accumulation of Noxa in ΔUBXD8 cells sensitizes these cells to apoptosis. Does Noxa over-expression cause a similar phenotype?

Typos should be corrected:
line 61: should be "regulate" instead of "regulates"
line 234: "cullin5" instead of "culin5"
line 258: "ubiquitination" instead of "uibiquitination"

A quantification of "mitolysosomes" should be provided in Figure 5B.

Referee #3:

This manuscript investigates UBXD8 and its roles in mitochondria function, in particular the links to mitophagy. Using fluorescent microscopy, it is shown that UBXD8 displays dual localization to the ER and Mitochondria. Moreover, UBXD8 depletion resulted in lower p97 association with mitochondria. These data are consistent with previous work in yeast. Generating a chmeric construct in which UBXD8 membrane region was replaced with the one of yeast FZO1, a mitochondria protein in yeast. Based on microscopy this protein appears to localize to mitochondria and facilitate the degradation of both mitochondrial and ER substrates. In contrast with work in yeast, in mammalian mitochondria UBXD8 does not seem to contribute for translocation associated degradation, even if UBXD8 associated with components of the TOM complex. To understand the function of UBXD8 in mitochondria the authors screened a variety of stress conditions. Using this approach, they found that loss of UBXD8 sensitizes cells to apoptosis, presumably due to the accumulation of pro-death BH3-only proteins such as NOXA, Bnip3 and BIK. Understanding the mechanism by which the ubiquitin proteasome system and p97-associated factors contribute to mitochondria homeostasis are important open questions. Moreover, some of the imaging data presented in this manuscript is interesting. However, the characterization of UBXD8 mitochondrial localization and function is only performed superficially. In my opinion, more extensive and rigorous analysis of UBXD8 localization requires quantification and further validation using biochemical

fractionation. Importantly these should include marker proteins and specificity controls.

Main points
1- Most experiments are poorly controlled. For example fractionation in Fig 1F
2- Microscopy experiments should be quantified and localization of UBXD* and mito-UBXD8 should be confirmed by biochemical methods. Based on the data presented, it is impossible to exclude that some mito-UBXD8 is in the ER.
3- Why are cells treated with -20*C MeOH prior fixation? I am concerned that such a harsh treatment for 30 minutes compromises the integrity of organellar membranes.
4- In Fig. 3 A-D, G Fig. 4 D, G, the authors used Titer-Glo Luminescent assay kit (Promega, G7570) to count number of alive cells, but if the author's model was correct deletion of UBXD8 would cause some mitochondrial phenotypes not only apoptosis and mitophagy but also OXPHOS activities. So, the authors should not use ATP concentration to estimate number of alive cells in these experiments. Alternatively, the authors should use FACS by Hochest33342 vs PI staining for total cells and dead cells, and by PI and Annexin V to know apoptosis process.
5- The quality of the blots for NOXA, Bnip3 and BIK can be improved. And the results should be quantified. Also, is there any evidence that UBXD8 can work together with Culin5?
6- In Fig. 5, steady-state mitophagy phenotype in UBXD8 knockout cells are not so strong. So, to avoid possibilities of side effects, the authors should use Breferdin A. Also gating shape are different between Fig. 5 C and E. What these experiments performed under dfferent conditions?

Minor points
1. In Fig. 2, 4, S1 S2, the CHX chase experiments should be quantified.
2. In Fig. S3 figure legend, "UBXD8 isdipensable for" should be "UBXD8 is dispensable for".

Referee #1:

In this manuscript, Zheng et al. report the role of UBXD8, an adaptor of the mammalian AAA-ATPase VCP, in mitochondria-associated degradation (MAD) of proteins acting in apoptosis and mitochondria-specific autophagy (mitophagy). VCP is an evolutionarily conserved multi-tasking enzymes critical for unfolding and extracting proteins from large complexes and organelles. In yeast, the VCP homolog Cdc48 interacts with Ubx2, an adaptor that is anchored to mitochondria and the endoplasmic reticulum (ER), promoting MAD and ER-associated degradation (ERAD), respectively. Whether VCP also mediates MAD in mammals remains unknown. Through a proteomics approach for ubiquitinated mitochondrial proteins and their interacting partners, the authors identified UBXD8 that has been suggested to act in VCP-mediated ERAD and lipid droplet-associated degradation. They performed a series of immunofluorescence imaging and cell fractionation, and found that UBXD8 also localizes to mitochondria and facilitates recruitment of VCP to mitochondria. In addition, mitochondria- and ER-resident ubiquitin E3 ligases co-immunoprecipitated with UBXD8. Cells lacking UBXD8 accumulated the mitochondrial proteins MiD49 and Mcl-1, and the ER protein Insig1. Interestingly, exclusively mitochondria-anchored UBXD8 variant (mito-UBXD8) restored efficient degradation of Mcl-1 and Insig1, indicating that the MAD system promotes degradation of substrates on both mitochondria and the ER. Additional results further suggest that UBXD8 acts in protection of cells against inducers of mitochondrial stress and apoptosis, and suppression of mitophagy and apoptosis via degradation of Bnip3 and Noxa, respectively.

The data in this study are well-organized and could provide new insights into the molecular mechanisms of MAD involving VCP and UBXD8 in mammals. However, it remains unclear if ER-localized VCP and UBXD8 promotes both ERAD in cis and MAD in trans. In addition, whether MAD requires UBXD8-dependent recruitment of VCP to mitochondria has not been investigated. In conclusion, this study would significantly be strengthened if the authors clarify these issues and address the following points.

Specific points:

1. In Figure 1F, the authors should perform a subcellular fractionation assay to examine fractions of whole cell homogenate, cytosol, mitochondria, and the ER by immunoblotting with antibodies specific for UBXD8, VCP, and representative markers. Note that the assay requires density gradient ultracentrifugation to separate

mitochondria from the ER.

We thank the referee for the constructive comments and suggestions. We have tested several protocols, including density gradient ultracentrifugation, to purify mitochondria and ER fractions. We finally used a protocol (**described in the methods section, line 431-469**) that does not require density gradient ultracentrifugation, because ultracentrifugation did not further improve organelle purity (data not shown).

We present the fractionation results in **Figure 2D and 2E**. Because of the strong tethering between mitochondria and the ER (*Phillips & Voeltz, Nat Rev Mol Cell Biol 2016*), the mitochondria and ER fractions contain residual ER and mitochondria contaminations (**Figure 2D**). Quantitative analysis shows that mitochondrial and ER VCP levels are significantly decreased by UBXD8 knockout, significantly increased by UBXD8 overexpression, and not affected by overexpressing the UBXD8-UBX* mutant (**Figure 2E**).

2. In Figure 2B, the authors should repeat the immunoprecipitation assays to clearly detect UBXD8 and VCP co-precipitated with 3HA-MARCH5 and 3HA-MUL1. It would also be better to test if the endogenous UBXD8 can be co-immunoprecipitated with the endogenous VCP, MARCH5, and RNF185.

We thank the referee for the suggestion. We noticed that in **Figure 2B**, the original UBXD8 western blot is too weak and have replaced it with a western blot of longer exposure time.

We fully agree with the referee that the examination of the interaction between endogenous UBXD8, VCP, and ubiquitin E3 ligases are critical. We used a FLAG-UBXD8 knockin HEK293T cell (endogenous UBXD8 level) to perform anti-FLAG IP. We normally used IP protocol with 1% Triton X-100 (**described in line 472-482**), but could not detect interaction between UBXD8 and VCP at normal condition (**Figure 2B**). With an improved IP protocol with 0.2% Triton X-100 (**described in line 483-493**), FLAG-UBXD8 pulled down endogenous VCP, MARCH5, and RNF185 at both normal and MG132-treated conditions (**Figure 2C**).

3. Exclusively ER-anchored UBXD8 (ER-UBXD8) and mito-UBXD8 should be expressed in cells lacking the endogenous UBXD8 and examined as described in Figures 2G-H, 4A, 4C, and 5B-D.

We have previously generated mito-UBXD8 and ER-UBXD8 but only showed the results of mito-UBXD8. Now we have provided results for both UBXD8 variants. We presented the construct design (**Figure 4A**) and examined their localization by fractionation (**Figure 4B**) and by immunofluorescence (**Figure 4C**). The fractionation experiment demonstrated the greatly improved mitochondrial localization of mito-

UBXD8 and improved ER localization of ER-UBXD8. But due to the issue of mitochondria-ER tethering, the fractionation data is not clean and not conclusive. We thus performed imaging analysis to demonstrate the exclusive mitochondrial localization of mito-UBXD8 and ER localization of ER-UBXD8 (**Figure 4C**). Both mito-UBXD8 and ER-UBXD8 can mediate the degradation of Mcl1 (mitochondrial substrate) and Insig1-FLAG (ER substrate) (**Figure 4D and 4E**). Similarly, both mito-UBXD8 and ER-UBXD8 can mediate the degradation of Noxa, Bnip3, and Bik (**Figure EV4B and EV4C**), and inhibit apoptosis (**Figure EV3D**) and mitophagy (**Figure 7E and 7F**). These new results consistently support that UBXD8 can work *in cis* and *in trans* to degrade mitochondrial and ER substrates.

4. The authors should quantify the protein levels analyzed in Figures 2C-E, 2H, 4A, and 4C.

We thank the referee for the suggestion. We have performed experiments in three biological repeats, quantified proteins levels, and calculated statistical significance in fractionation experiments (**Figure 2E**) and all the CHX chasing experiments (**Figure 3B, 3D, 3F, 4E, 6B, 6C, EV1B, EV2B, EV4C, EV4E, and EV4G**).

5. UBX*, a UBXD8 mutant defective in VCP binding (Figure S2A), should be expressed in cells lacking the endogenous UBXD8 and examined as described in Figures 1F-G, 2H, 4A, 4C, and 5B-D. It would also be great to generate mito-UBX* and ER-UBX*, and analyze them as described in Figures 2G-H, 4A, 4C, and 5B-D.

We thank the referee for the suggestion. We have analyzed the role of UBX* in VCP recruitment to mitochondria and the ER (**Figure 2D and 2E**), apoptosis (**Figure EV3E**), mitophagy (**Figure 7E and 7F**), and the degradation of Noxa, Bik, and Bip3 (**Figure EV4D and EV4E**).

We kindly wish the referee agree with us that experiments with ΔUBXD8+mito-UBX* and ΔUBXD8+ER-UBX* are not necessary because the above-mentioned experiments have clearly demonstrated UBX* is not functional.

6. In Figure 5B-C, the authors should investigate autophagy-defective mutants lacking UBXD8 and demonstrate that an increase in mitophagy-positive signals by loss of UBXD8 indeed depends on autophagy.

We have knocked down autophagy gene Beclin1 to block mitophagy in ΔUBXD8 cells (**Figure EV5C and EV5D**).

Referee #2:

In the present manuscript Zheng et al. investigate a putative mitochondrial function of

the protein UBXD8, previously described to function at the endoplasmic reticulum and lipid droplets. The authors localize a fraction of UBXD8 to mitochondria, and present data pointing to a UBXD8 dependent recruitment of VCP to mitochondria. They identify several mitochondrial substrates of UBXD8 and thereby link the function of UBXD8 to apoptosis and mitophagy. The manuscript provides potentially very interesting insights into the turnover of proteins at the mitochondrial outer membrane. However, additional data are required to show the specificity of the findings.

Major points:

1. The localization of UBXD8 is of central importance for the conclusions of this manuscript. The authors used fluorescence microscopy to determine the cellular localization of UBXD8. The authors should also utilize an alternative methods like cellular fractionation to show the localization of endogenous UBXD8. The authors may also show the mass spectrometry data of mitochondrial proteins to reveal how they identified UBXD8 in a mitochondrial fraction.
2. In Figure 1F the authors present isolated mitochondria, in which VCP is reduced in absence of UBXD8 suggesting that UBXD8 recruits VCP to mitochondria. Given the normally high abundance of ER in a mitochondrial preparation, the authors should show an ER protein as control.

We thank the referee for the constructive comments and suggestions. Questions 1 and 2 are similar questions. We thus address them together.

We have performed fractionation analysis of mitochondrial and ER fractions to demonstrate UBXD8 recruits VCP to mitochondria and the ER (**Figure 2D and 2E**).

For the mass-spectrometry data, it is an experiment performed more than 10 years ago. In that experiment, we induced apoptotic mitochondrial damage (by tBid) and simultaneously applied caspase inhibitor to keep cell viability. We observed extensive ubiquitination of damaged mitochondria. We thus generated an HA-ubiquitin stable line to immunoprecipitated ubiquitinated proteins from control and damaged mitochondrial fractions. We cut ~10 bands from the silver staining gel for mass-spec analysis. Below are one pair of the bands from control and tBid-damaged mitochondria. UBXD8 (FAF2) appeared in the control sample (**yellow**) but disappeared after tBid damage. We quickly observed the mitochondrial localization of UBXD8 but had a hard time to reveal its mitochondrial function. It sat on the shelf for years before we repicked it up. Because it is a long and twisted story that is not directly related to the current study, I would like

to make it clear here but not to include it into the manuscript.

Sample ID	Accession number	Protein description	Mascot score	MW
cont2_4	IPI00645452	Tax_Id=9606 Gene_Symbol=TUBB Tubulin, beta	599	47736
	IPI00294619	Tax_Id=9606 Gene_Symbol=TFG Protein TFG	592	43421
	IPI00007752	Tax_Id=9606 Gene_Symbol=TUBB2C Tubulin beta-2C chain	487	49799
	IPI00013683	Tax_Id=9606 Gene_Symbol=TUBB3 Tubulin beta-3 chain	397	50400
	IPI00031801	Tax_Id=9606 Gene_Symbol=CSDA Isoform 1 of DNA-binding protein A	361	40066
	IPI00303476	Tax_Id=9606 Gene_Symbol=ATP5B ATP synthase subunit beta, mitochondrial	344	56525
	IPI00172656	Tax_Id=9606 Gene_Symbol=FAF2 FAS-associated factor 2	332	52591
tBid2_4	IPI00303476	Tax_Id=9606 Gene_Symbol=ATP5B ATP synthase subunit beta, mitochondrial	802	56525
	IPI00294619	Tax_Id=9606 Gene_Symbol=TFG Protein TFG	378	43421
	IPI00645452	Tax_Id=9606 Gene_Symbol=TUBB Tubulin, beta	365	47736
	IPI00784154	Tax_Id=9606 Gene_Symbol=HSPD1 60 kDa heat shock protein, mitochondrial	347	61016
	IPI00007752	Tax_Id=9606 Gene_Symbol=TUBB2C Tubulin beta-2C chain	312	49799
	IPI00013683	Tax_Id=9606 Gene_Symbol=TUBB3 Tubulin beta-3 chain	266	50400
	IPI00102997	Tax_Id=9606 Gene_Symbol=WRNIP1 Isoform 2 of ATPase WRNIP1	243	69416
	IPI00013881	Tax_Id=9606 Gene_Symbol=HNRNPH1 Heterogeneous nuclear ribonucleoprotein H	200	49198

3. Are the identified mitochondrial UBXD8 substrates involved in mitophagy and apoptosis degraded in a VCP dependent manner? Does the VCP-binding deficient variant UBXD8 UBX* as presented in Figure S2A show increased sensitivity to apoptosis and increased autophagy rates?

We thank the referee for the great question. We have performed VCP knockdown to show that the degradation of the newly-identified UBXD8 substrates is VCP dependent (**Figure EV4F and EV4G**). The UBXD8-UBX* mutant could not rescue the impaired degradation of Noxa, Bnip3, and Bik (**Figure EV4D and EV4E**), the increased apoptosis sensitivity (**Figure EV3E**) and mitophagy level (**Figure 7E and 7F**) in ΔUBXD8 cells.

4. The authors report that mitochondrial UBXD8 mediates the turnover of the ER protein Insig1-FLAG, while ER resident UBXD2 also mediates turnover of the mitochondrial protein Mid49. To exclude mislocalization of the substrate proteins, it would be important to assess the subcellular localization of Insig1-FLAG and Mid49 in UBXD8 and UBXD2 deficient cells.

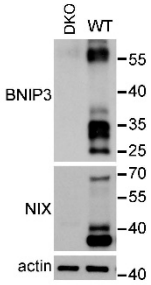
We have examined the subcellular localization of MiD49-FLAG **(Figure 3G**) and Insig1-FLAG (**Figure EV2C**) in WT, ΔUBXD8, and ΔUBXD2 cells. MiD49 localizes to mitochondria and Insig1 localizes to the ER in all three cells. Together with the mito-UBXD8 and ER-UBXD8 results (**Figure 4**), our results highlight that mito- and ER-VCP adaptors can work *in cis* and *in trans* to mediate substrate degradation.

5. The authors observe increased sensitivity of cells lacking UBXD8 to actinomycin D and doxorubicin, which they link to increased apoptosis. The authors should provide additional data to support this conclusion like cytochrome c release. Furthermore, it is not entirely clear whether Bnip3 is degraded in a UBXD8-dependent manner. The

immunostainings in Figure 4 for Bnip3 and Nix mark several bands. It remains unknown which is the specific signal.

We have provided additional data to support that ΔUBXD8 cells have increased apoptosis sensitivity. First, ΔBAX ΔBAK cells are completely resistant to cell death induced by actinomycin D and Doxorubicin (**Figure EV3A**). Second, we performed FACS analysis to show that ΔUBXD8 cells have increased PS externalization (Annexin V-positive) and membrane breakage (PI-positive) (**Figure EV3B and EV3C**).

For the immunostaining of Bnip3 and Nix, both proteins exist in monomer, dimer (resistant to SDS denaturing), and modified forms. All the bands are true signals. The specificity of the Bnip3 and Nix antibodies has been verified by knockout cells (**see below**). The specificity of the Bnip3 antibody can be seen from the western blot of ΔBNIP3 cells (**Figure 6D and 6F**).



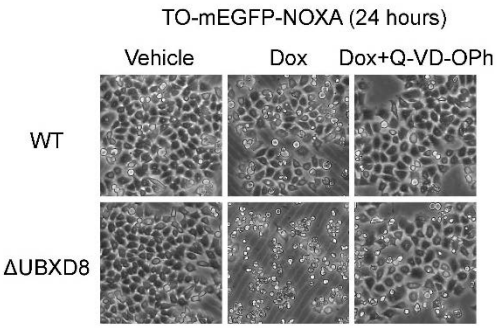
Minor points:

Line 305: The authors propose that UBXD8 is an essential MAD component. This needs to be rephrased as UBXD8 is not essential for cell survival. The loss of UBXD8 does not abrogate degradation of the identified substrates, indicating further quality control pathways. Thus, UBXD8 is not an essential MAD component.

We have replaced “an essential MAD component” as “an important MAD component” (**line 334**).

The authors state that the accumulation of Noxa in ΔUBXD8 cells sensitizes these cells to apoptosis. Does Noxa over-expression cause a similar phenotype?

As shown below, we inducibly expressed EGFP-Noxa for 24 hours and potently induced cell death in ΔUBXD8 cells, which was inhibited by the caspase inhibitor Q-VD-Oph.



Typos should be corrected:
line 61: should be "regulate" instead of "regulates"
line 234: "cullin5" instead of "culin5"
line 258: "ubiquitination" instead of "uibiquitination"
We thank the referee for the careful examination. We have corrected these typos.

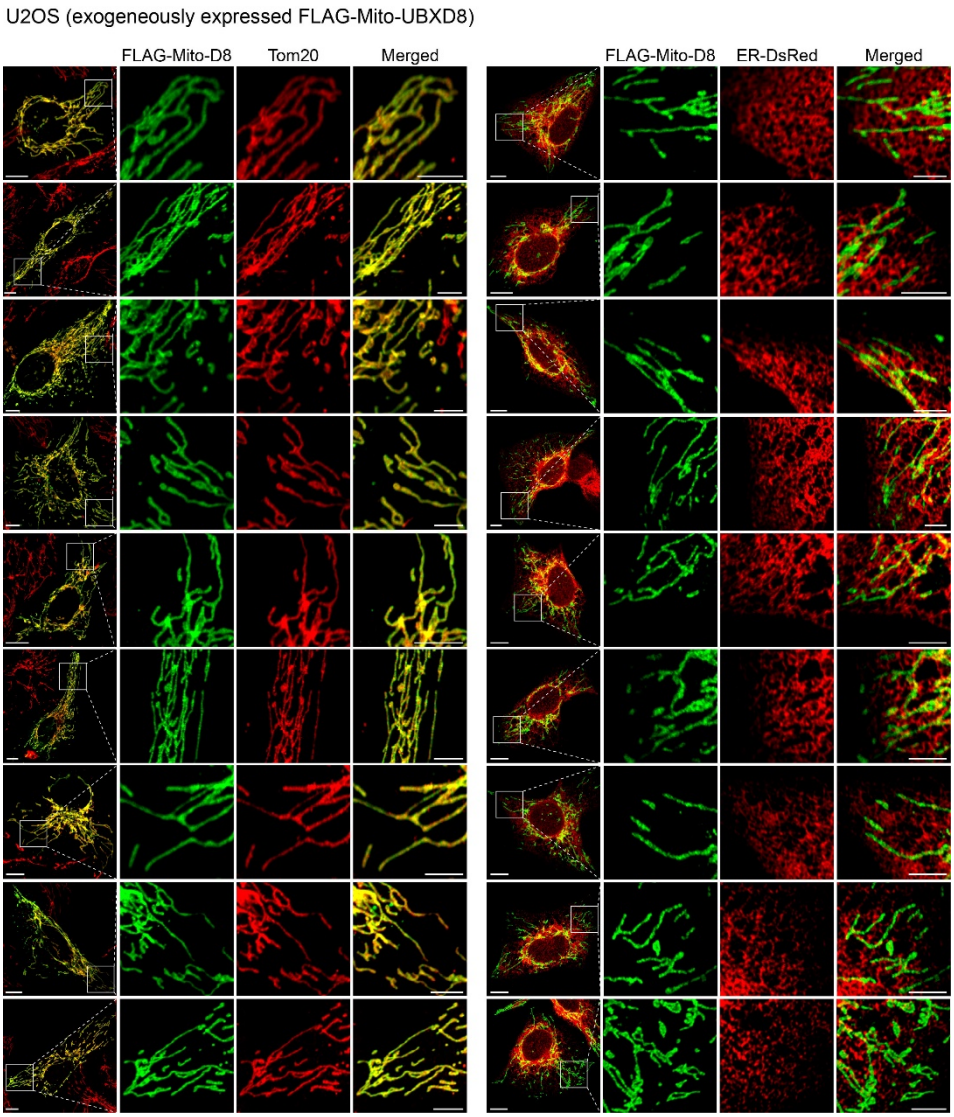
A quantification of "mitolysosomes" should be provided in Figure 5B.
We have provided quantification of mitolysosomes in **Figure 7C and 7D**.

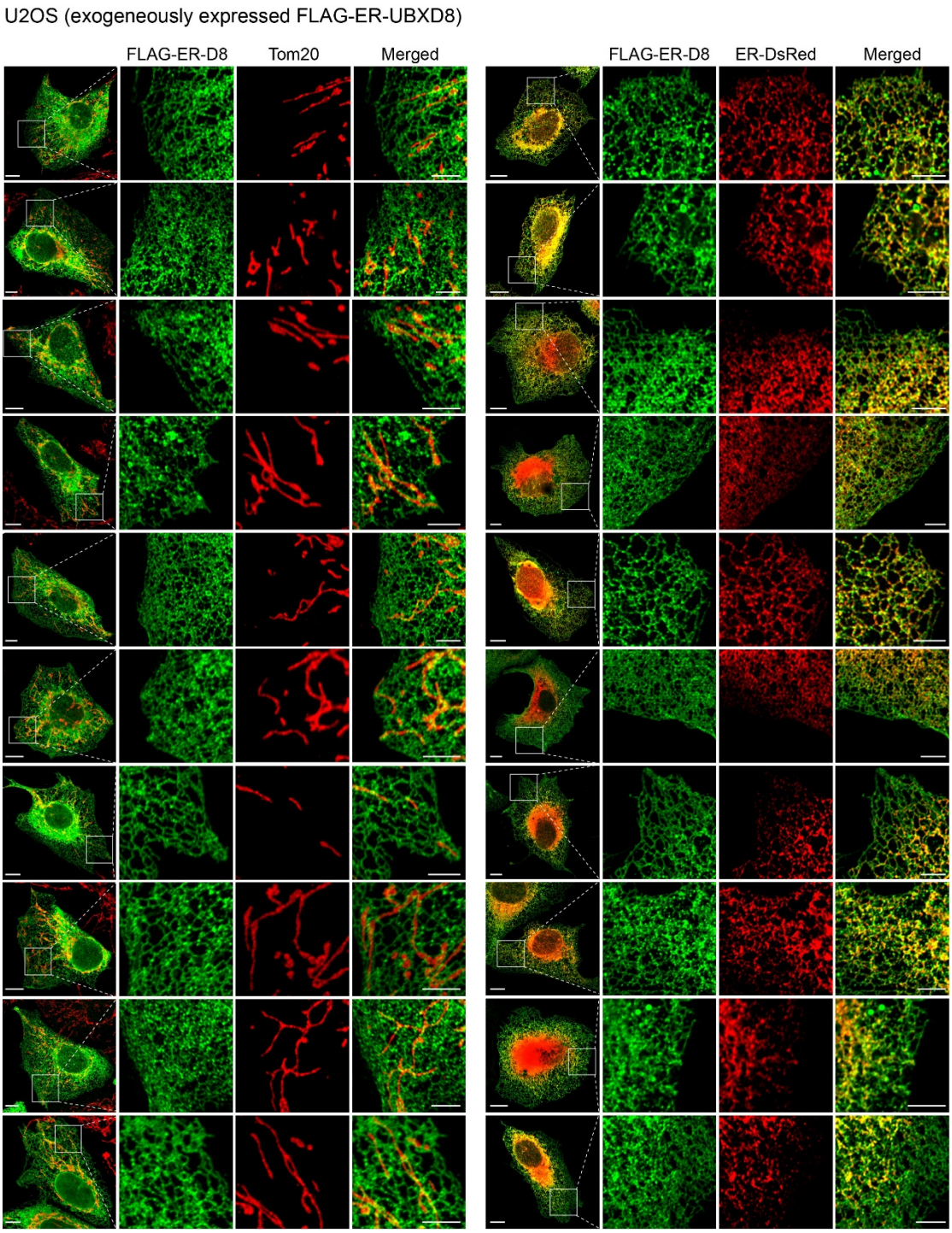
Referee #3:
This manuscript investigates UBXD8 and its roles in mitochondria function, in particular the links to mitophagy. Using fluorescent microscopy, it is shown that UBXD8 displays dual localization to the ER and Mitochondria. Moreover, UBXD8 depletion resulted in lower p97 association with mitochondria. These data are consistent with previous work in yeast. Generating a chmeric construct in which UBXD8 membrane region was replaced with the one of yeast FZO1, a mitochondria protein in yeast. Based on microscopy this protein appears to localize to mitochondria and facilitate the degradation of both mitochondrial and ER substrates. In contrast with work in yeast, in mammalian mitochondria UBXD8 does not seem to contribute for translocation associated degradation, even if UBXD8 associated with components of the TOM complex. To understand the function of UBXD8 in mitochondria the authors screened a variety of stress conditions. Using this approach, they found that loss of UBXD8 sensitizes cells to apoptosis, presumably due to the accumulation of pro-death BH3-only proteins such as NOXA, Bnip3 and BIK.
Understanding the mechanism by which the ubiquitin proteasome system and p97-associated factors contribute to mitochondria homeostasis are important open questions. Moreover, some of the imaging data presented in this manuscript is interesting. However, the characterization of UBXD8 mitochondrial localization and function is only performed superficially. In my opinion, more extensive and rigorous analysis of UBXD8 localization requires quantification and further validation using biochemical fractionation. Importantly these should include marker proteins and specificity controls.

Main points
1- Most experiments are poorly controlled. For example, fractionation in Fig 1F.
We thank the referee for the constructive comments and suggestions. We have re-performed the fractionation experiments and probed the marker proteins for mitochondria, ER, and the cytosol (**Figure 2D and 2E**).

2- Microscopy experiments should be quantified and localization of UBXD* and mito-UBXD8 should be confirmed by biochemical methods. Based on the data presented, it is impossible to exclude that some mito-UBXD8 is in the ER.

We have performed fractionation analysis of UBXD8* (**Figure 2D and 2E**), mito-UBXD8, and ER-UBXD8 (**Figure 4B**). **Figure 4B** demonstrated the greatly improved mitochondrial localization of mito-UBXD8 and improved ER localization of ER-UBXD8. But because of the strong tethering between mitochondria and the ER (*Phillips & Voeltz, Nat Rev Mol Cell Biol 2016*), the mitochondria and ER fractions contain residual ER and mitochondria contaminations and the fractionation analysis is not conclusive. We thus performed immunofluorescence analysis to demonstrate the exclusive mitochondrial localization of mito-UBXD8 and ER localization of ER-UBXD8 (**Figure 4C**). We have carefully examined tens of cells. Some additional examples are shown here.





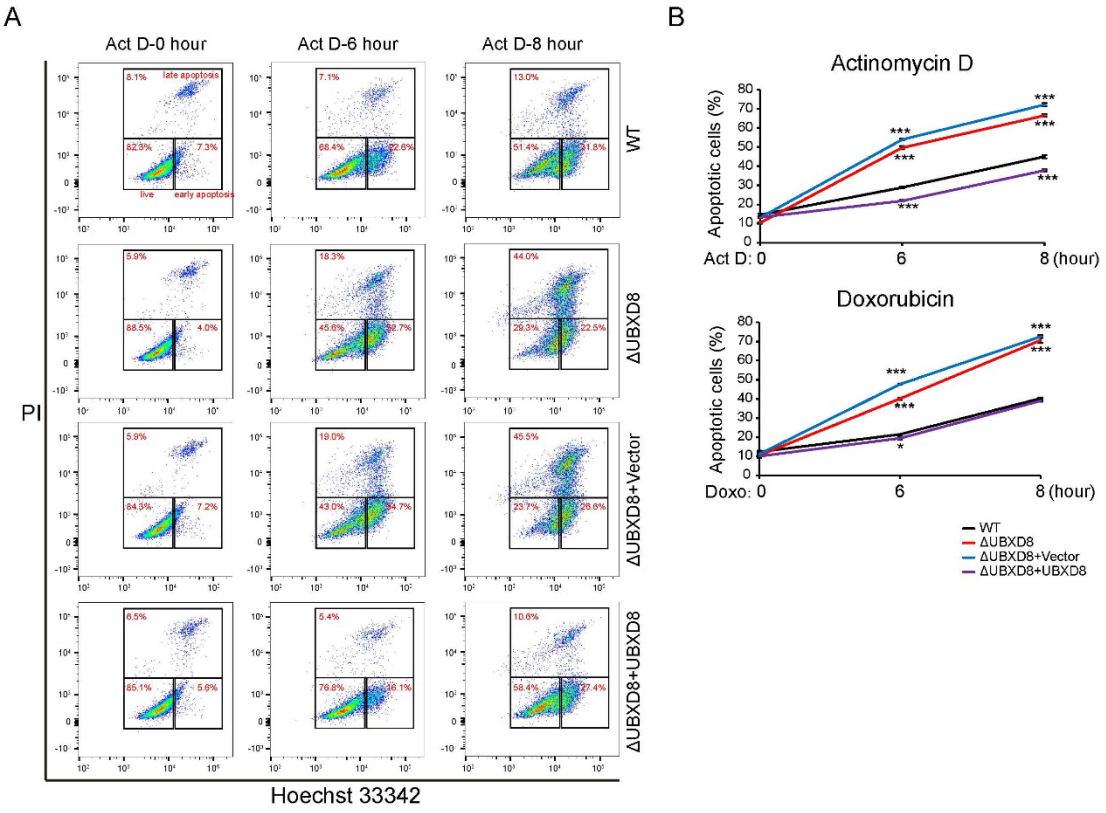
3- Why are cells treated with -20*C MeOH prior fixation? I am concerned that such a harsh treatment for 30 minutes compromises the integrity of organellar membranes.

This method was only used in **Figure 1C**. Because immunofluorescence staining with the FLAG M2 antibody has a certain level of background signal, which is OK for FLAG-tagged proteins with high expression level but causes problems for those with low expression level, such as endogenously-tagged FLAG-UBXD8. We thus used -20℃

MeOH to release the cytosol to reduce the background signal before fixation.

4- In Fig. 3 A-D, G Fig. 4 D, G, the authors used Titer-Glo Luminescent assay kit (Promega, G7570) to count number of alive cells, but if the author's model was correct deletion of UBXD8 would cause some mitochondrial phenotypes not only apoptosis and mitophagy but also OXPHOS activities. So, the authors should not use ATP concentration to estimate number of alive cells in these experiments. Alternatively, the authors should use FACS by Hochest33342 vs PI staining for total cells and dead cells, and by PI and Annexin V to know apoptosis process.

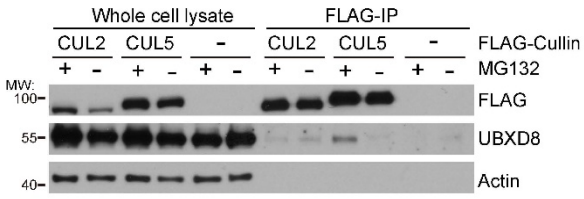
We thank the referee for the great suggestions. We have performed FACS analysis with Hochest33342 & PI, and with PI & Annexin V as suggested. We showed the PI & Annexin V result to demonstrate ΔUBXD8 cells have increased apoptosis sensitivity (**Figure EV3B and EV3C**). The Hochest33342 & PI results (**shown below**) are similar to PI & Annexin V results. Due to space limitation, we did not add them to Figure EV3. In addition, we would like to explain that beyond using Titer-Glo to measure cell viability, we also took images for all the experiments related to cell death as shown in **Figure 5D**. They are consistent with the Titer-Glo results. We did not show all the images because of space limitation.



5- The quality of the blots for NOXA, Bnip3 and BIK can be improved. And the results should be quantified. Also, is there any evidence that UBXD8 can work together with Culin5?

Thanks for the suggestion. We have performed experiments in three biological repeats, quantified proteins levels, and calculated statistical significance in fractionation experiments (**Figure 2E**) and all the CHX chasing experiments (**Figure 3B, 3D, 3F, 4E, 6B, 6C, EV1B, EV2B, EV4C, EV4E, and EV4G**).

To examine the interaction between UBXD8 and Cullin5, we expressed FLAG-tagged Cullin2 and Cullin5, and performed anti-FLAG IP. We found that UBXD8 associates with Cullin5 under MG132 treatment (**shown below**).



6- In Fig. 5, steady-state mitophagy phenotype in UBXD8 knockout cells are not so strong. So, to avoid possibilities of side effects, the authors should use Breferdin A. Also gating shape are different between Fig. 5 C and E. What these experiments performed under different conditions?

We have knocked down the autophagy gene Beclin1 and blocked mitophagy in ΔUBXD8 cells (**Figure EV5C and EV5D**). Thus, the steady-state mitophagy phenotype in ΔUBXD8 cells is truly due to the autophagic degradation of mitochondria. The different gating shapes in Fig. 5C and 5E (**now Figure 7G and 7L**) may be because: 1. The experiments were performed on different FACS machines, which may cause variation. 2. The mitoKeima reporter may have different expression levels in Fig. 5C and 5E because they were generated at different time and with different baches of lentivirus expressing mitoKeima.

Minor points

1. In Fig. 2, 4, S1 S2, the CHX chase experiments should be quantified.

We have quantified all the CHX chasing experiments (**Figure 3B, 3D, 3F, 4E, 6B, 6C, EV1B, EV2B, EV4C, EV4E, and EV4G**).

2. In Fig. S3 figure legend, "UBXD8 isdipensable for" should be "UBXD8 is dispensable for".

We have corrected the error.

Dear Dr. Jiang

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that was asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your work. Referees #1 and #3 have remaining concerns or suggestions to improve the manuscript I ask you to address in a final revised version of the manuscript. Please also provide a final response to the referee points and comment on the report of referee #3.

Moreover, I have these editorial requests I also ask you to address in a final revised manuscript:

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- Please add a formal 'Data Availability Section' after the Methods. This is now mandatory, like the conflict of interest statement. If no primary datasets have been deposited, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

- Please order the manuscript sections like this, using these names:
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- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to the microscopic images (main, EV and Appendix figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, the scale bars for many images are too thin or are hard to see (please change their colour to white) or are even missing.

- As the Western blots shown are significantly cropped, please provide the source data for the blots. 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. Please submit the source data for all the Western blots shown in the main and EV figures (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure.

- Please move Tables S1 and S2 in a single pdf file labeled Appendix and upload this as 'Expanded View Content'. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Table S1 and Appendix Table S2 for the tables throughout the text, and also label the tables according to this nomenclature. Finally, please remove the tables from the main manuscript text.

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- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

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Senior Editor
EMBO Reports

Referee #1:

In this revised manuscript, the authors provided additional data and descriptions to address most of the issues pointed by the referees. In particular, the data from subcellular fractionation assays for separating mitochondria and the ER will support the novel point that UBXD8 is localized on both organelles, recruiting VCP to act in degradation of a broader range of substrates. Moreover, the findings that artificially mitochondria- or ER-localized UBXD8 can promote degradation of ER or mitochondrial resident proteins, respectively, advocate that MAD and ERAD may function in cis and trans. Overall, the revised version is intriguing and could provide new mechanistic insights into the protein quality control pathways, and the arranged data set is in much nicer conditions. These improvements, in addition to clarifying some minor points below, could help this study to be accepted for publication in EMBO Reports.

Minor points
1. In Figure 4D, the authors should mention that WT-, Mito-, and ER-UBXD8 are expressed in UBXD8 knock-out HeLa cells as described in Figure 4B (with the protein levels similar to the endogenous UBXD8), if that is indeed the case. Why are the levels of Mito-UBXD8 much lower than those of WT- and ER-UBXD8?

2. The discussion section would be more stimulating if the authors could propose possible physiological benefits of UBXD8-mediated mitochondria-ER crosstalk in protein degradation pathways.

Referee #2:

THe authors addressed all my concerns in the revised version of the manuscript. I recommend publication of the manuscript in EMBO Report.

Referee #3:

The revised manuscript has some improvements, in particular the inclusion of the fractionation experiments which support the conclusion that UBXD8 has dual localization to the ER and mitochondria.

The fact that both ER and Mitochondria localized UBXD8 are functional for ER and mitochondrial substrates is confusing and counterintutive as it suggests that UBXD8 localization is irrelevant for its function. Also it is surprising that MITO-UBXD8 is fully functional considering that it has such a short half-life (Fig 4D).

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We sincerely thank the referee for the suggestion. We have described cell line generation in **Figure 4D legend** as suggested.

The reason that mito-UBXD8 has much lower expression level than WT- and ER-UBXD8 is not entirely clear. From the CHX chasing experiment in **Figure 4D**, we can see that mito-UBXD8 is less stable (has much shorter half-life) than the other two forms. It is likely that mito-UBXD8 can be recognized and degraded by some mitochondrial or cytosolic E3 ubiquitin ligases.

2. The discussion section would be more stimulating if the authors could propose possible physiological benefits of UBXD8-mediated mitochondria-ER crosstalk in protein degradation pathways.

Thanks for the suggestion. We have expanded the discussion of the crosstalk of MAD and ERAD in the last paragraph of the **Discussion** section (**line 347-363**).

The discussion is as follows:

> Our study also reveals the intimate crosstalk between MAD and ERAD. We show that ER-localized VCP adaptor UBXD2 participates in MAD (**Figure 3**), and both mitochondria- and ER-localized UBXD8 can mediate MAD and ERAD (**Fig 4**). The cross-membrane degradation of substrates most likely occurs at the mitochondria-ER contact site, where mitochondria and the ER juxtapose at a distance of ~10-50 nm (Csordás *et al*, 2006; Giacomello & Pellegrini, 2016; Murley & Nunnari, 2016a; Wang *et al*, 2015). Considering that a ubiquitin molecule has a diameter of ~3.4 nm and the VCP hexamer has a diameter of ~20 nm (Halawani *et al*, 2009), it is easy for poly-ubiquitinated membrane proteins to be reached by the VCP complex on the opposite membrane at the

mitochondria-ER contact site. The close cooperation between MAD and ERAD may facilitate the degradation of a subset of mitochondrial/ER substrates that localize to or can diffuse into the contact site. However, because not all the ER and mitochondria are in contact, and because the ER is a highly compartmentalized organelle with subdomains different in protein composition and function (Lynes & Simmen, 2011), we speculate that MAD and ERAD cannot compensate each other for all the substrates, which necessitates mitochondria/ER-resident adaptor-VCP complexes.

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We sincerely thank the referee for raising this issue. We have added a paragraph in the **Discussion** section (**line 347-363**) to discuss the crosstalk between MAD and ERAD. The discussion is as follows:

Our study also reveals the intimate crosstalk between MAD and ERAD. We show that ER-localized VCP adaptor UBXD2 participates in MAD (**Figure 3**), and both mitochondria- and ER-localized UBXD8 can mediate MAD and ERAD (**Fig 4**). The cross-membrane degradation of substrates most likely occurs at the mitochondria-ER contact site, where mitochondria and the ER juxtapose at a distance of ~10-50 nm (Csordás *et al*, 2006; Giacomello & Pellegrini, 2016; Murley & Nunnari, 2016a; Wang *et al*, 2015). Considering that a ubiquitin molecule has a diameter of ~3.4 nm and the VCP hexamer has a diameter of ~20 nm (Halawani *et al*, 2009), it is easy for poly-ubiquitinated membrane proteins to be reached by the VCP complex on the opposite membrane at the mitochondria-ER contact site. The close cooperation between MAD and ERAD may facilitate the degradation of a subset of mitochondrial/ER substrates that localize to or can diffuse into the contact site. However, because not all the ER and mitochondria are in contact, and because the ER is a highly compartmentalized organelle with subdomains different in

protein composition and function (Lynes & Simmen, 2011), we speculate that MAD and ERAD cannot compensate each other for all the substrates, which necessitates mitochondria/ER-resident adaptor-VCP complexes.

We speculate that the substrates analyzed in this manuscript (MiD49/Mcl1/Bnip3/Noxa/Insig1) happen to be those that localize to or can diffuse into the contact site and can be degraded by mitochondria- and ER-tethered VCP. The degradation of ubiquitinated substrates on organelles without contact sites or at organelle subdomains distant to the contact sites most likely requires organelle-resident VCP complexes. Identification and characterization of more MAD and ERAD substrates will help address this issue.

Dr. Hui Jiang
National Institute of Biological Sciences, Beijing
No. 7, Science Park Road, Zhongguancun Life Science Park
Beijing 102206
China

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods, Table S1 and Table S2

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures and Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	figure legend
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	figure legend

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
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Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	