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# ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy

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**Review timeline:**

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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.)

## Transfer Note:

Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed. It was then transferred to EMBO reports with the original referees comments attached.

Editor: Nonia Pariente

Presubmission Enquiry - authors 07 November 2013

I am one of the finder of a mitochondrial protein, FUNDC1, a novel mitophagy receptor, and the first author of the paper on FUNDC1 (2012,NCB paper).

Recently, I submitted an interesting finding to the EMBO journal (We found that Fundc1 is a novel substrate of key autophagy kinase ULK1 and they coordinatedly regulate mitophagy), and it has been sent out for deep review. I got 2 positive and 1 negative feedbacks from the reviewers. I have addressed most of the concerns of these reviewers but one of them asked us to do some experiments

far beyond the scope of this paper.

I disscussed with Dr. Hong Zhang (HHMI early career scientist from Institute of Biophysics, Chinese Academy of Sciences) and he recommended that I should send the ms. for your consideration. I added more control experiments and new figures to the new ms. .

I would like to ask a possibility if we can submit to EMBO reports? Please take a look at it.

Attached please find the point-by-point answers, the text, and figures.

(Please see below under 'Authors reply to original referees comments: January 17)

Editorial Decision 12 November 2013

First of all, many thanks for considering EMBO reports for publication. Thanks also for your patience while I have found the time to assess your study, the referee reports and your responses to them.

Although the concept of phospho-regulated LIRs is not entirely new in autophagy, given the current interest in identifying ULK1 substrates and understanding the mechanisms of mitophagy, I think we could be interested in a fully revised manuscript, and agree that identifying how ULK1 recognizes damaged mitochondria is out of the scope of the present study.

We would nevertheless require that you perform some of the experiments requested by the referees, as detailed below. Please note that, if additional ULK1 substrates (especially linking to specific types of autophagy) were described in the meantime, we would consider this compromises the conceptual advance of the study.

We would request that the following experiments are included, in order to send the study to the referees (I am not sure from your letter if you are able/willing to provide them):

- The minimal binding region between ULK1 and FUNDC1 would have to be identified, and clarify whether FUNDC1 needs to be at mitochondria to interact with ULK1. Also, point mutants that disrupt ULK1-FUNDC1 interaction need to be constructed and used to shown that this interaction and Ser-17P is crucial for mitophagy.

- provide experimental answer to ref 2's point 6, analyzing the effect of proteasomal inhibition on TOM20/VDAC levels, and perform +/- bafilomycin experiments after 24 hours of exposure

- use an additional measure of mitochondrial abundance to bolster your claims, such as citrate synthase activity

- provide in the supplement a comprehensive analysis of the anti-FUNDC1 (ser17) antibody as a stand-alone figure

If no novelty concerns arise and the above issues have been addressed, I would then send the study to the same three referees, and alert the critical one that we do not require the elucidation of upstream ULK1 regulation. I think this would be the fastest way, as they are already familiar with the study.

I hope this sounds like a reasonable plan. Please let me know if anything is unclear and what your plans are. Also, if positive, an approximate timeline for submission would be nice.

Submission of revised manuscript to EMBO reports - authors reply to original referees comments - 17 January 2014

**Point to point responses to referees' comments**

Referee #1:

In this manuscript, Wu et al reported that ULK1 interacts and phosphorylates FUNDC1 on Ser17 and this phosphorylation is important for proper mitophagy induction. The study made an interesting connection between the mitophagy receptor protein and the core autophagic machinery, providing insights as to how mitophagy activity could be specifically regulated by autophagy proteins. The manuscript is on an intriguing topic but it seems premature at present. Several experiments need to be better controlled and the results clarified. Some important points relating with other studies need to be clearly discussed.
**Answer: Thanks for the invaluable comments. We added additional experiments and wish our answers would fully satisfy the referee.**

1. In Figure 1A-D, the authors observed the upregulation of ULK1 protein level upon hypoxia or FCCP treatment, suggesting ULK1 specifically regulates mitophagy. However, to make the point of specificity, the protein level change of ULK1 under other autophagy-inducing conditions, such as starvation, should be shown as a control. It is possible that the ULK1 protein level increase is responsive to any autophagic stimulus, not just mitophagy.
**Answer: This is a good suggestion. We examined the ULK1 protein level under starvation condition and found that ULK1 is mildly induced within 1 hour starvation but decreased after prolonged treatment (fig S1A). We did not observe the association of ULK1 with mitochondria under this condition (fig S1C).**

2. Figure 2 explored the interaction between ULK1 and FUNDC1. While Figure 2C showed the interaction is dependent on hypoxia or FCCP treatment, Figure 2 A and B were done under non-treated conditions. Is the tagged version of ULK1 and FUNDC1 overexpressed? Considering the inconsistency between Figure 2C and A/B, how would the authors evaluate the effect of protein overexpression (or tag) on their interactions for this experiment? Would stronger interaction be observed if the cells are treated with hypoxia or FCCP?
**Answer: Yes, the tagged version of ULK1 and FUNDC1 was overexpressed. In our previous paper, we have shown that FUNDC1 overexpression is a potent mitophagy stimulus (Liu, et al. Nat Cell Bio, 2012). The tagged version of ULK1 and FUNDC1 immunoprecipitation experiments were done under Fundc1 overexpression condition (where mitophagy is induced) in Figure 2A and B. Hypoxia or FCCP treatment is the established mitophagy-inducing condition too. So, the two proteins have endogenous interaction under hypoxia or FCCP treatment, while they have no interaction in non-treated cells or IgG control (Figure 2C).**

3. Figure 2C studied the interaction between the endogenous level of ULK1 and FUNDC1 and suggested they only interact under mitophagy-inducing conditions (although very weakly). However, as Figure1 shows, the ULK1 protein level was upregulated under these conditions (and Figure 2C also showed a higher level of ULK1 especially under FCCP treatment). How do the authors exclude the possibility that the observed interaction is due to higher ULK1 protein level?
**Answer: ULK1 has a considerable expression level even under basal condition (please see the first two input ULK1 bands in fig2C and the ULK1 bands at 0h in fig 2G, 2H), so the amount of lysed cellular ULK1 in IP experiment is enough for binding to FUNDC1. Therefore, we can exclude the possibility that the observed interaction is due to higher ULK1 protein level. Furthermore, in fig2C, although ULK1 had been successfully immunoprecipitated by its own antibody in untreated cells (IP band) there is no FUNDC1 band in IgG or untreated cells (because it is non-mitophagy-inducing condition). This strongly supports that the specific binding of ULK1 and FUNDC1 only happens under mitophagy inducing conditions (fig2B and 2C). *In vitro* kinase assay also verified their connection (fig2F).**

4. Add reference for ULK1 K46N or present evidence that it is kinase dead ULK1.

**Answer: We added reference for ULK1 K46N in the results and discussion section, and tested the phosphorylation activity of ULK1 K46N by using ATG13 as a substrate (It is an established substrate of ULK1). We found that ULK1 K46N is not completely kinase dead, since ATG13 S318 has minor phosphorylation signal (fig S4).**

5. In Figure 2 D and E, FUNDC1 still shows some level of phosphorylation with ULK1 K46N. Discussion should be provided for this point. Is it because the K46N mutant is not completely kinase dead, or is there another kinase(s) also involved in FUNDC1 phosphorylation? Also, since FUNDC1 is phosphorylated on other sites (see Liu et al, 2012), how specific is the anti-FUNDC1 (Ser17) antibody in vivo (Figure 2F is an in vitro experiment)?
**Answer: This is because ULK1 K46N still has minor kinase activity (fig S4, please also see the answer to point 4). We thoroughly tested the specificity of anti-FUNDC1 (Ser17) antibody. Anti-FUNDC1 (Ser17) can recognize FUNDC1 WT and FUNDC1 (S17D) but cannot recognize FUNDC1 (S17A), whereas anti-FUNDC1 (Tyr18) can recognize FUNDC1 (S17A) but cannot recognize FUNDC1 (S17D), because FUNDC1 (S17D) or FUNDC1 (WT) overexpression induces mitophagy where Ser-17 is phosphorylated and Tyr-18 is dephosphorylated (fig S3, fig S11B). The anti-FUNDC1 (Ser17) antibody cannot recognize overexpressed FUNDC1-Myc in ULK1 (-/-) cells (*in vivo*), which has Src expression, further supports its specificity (fig2E, lane 2).**

6. In Figure 2F, what are the other two bands on top of the GST band?
**Answer: We are sorry for the unclear description. They are GST-FUNDC1 (WT) and GST-FUNDC1 (S17A). GST is often co-expressed with GST-FUNDC1 (WT) and GST-FUNDC1 (S17A).**

7. Figure 3 characterized the domains inside FUNDC1 that are responsible for its interaction with ULK1. Amino acids 50-139 contain two transmembrane domains that are potentially important for the mitochondrial localization of FUNDC1 (for example, the 96-155 fragment seemed to localize in a more diffuse fashion). It is necessary to clarify which domains are important for the mitochondrial localization of FUNDC1 and whether the mitochondrial localization of FUNDC1 is required for its interaction with ULK1.
**Answer: We made a series of deletions and mutations and found that FUNDC1 lost its mitochondrial localization if the first and second transmembrane domains are simultaneously deleted (Because 1-139, 69-138, 50-71/97-155 localized to mitochondria while 96-155 does not) (fig 2I and S7). We also found that mitochondrial localization of FUNDC1 is necessary but not sufficient for its interaction with ULK1 because 1-96 has mitochondrial localization but does not interact with ULK1 (fig 2I and S7). This is because 1-96 lost the key ULK1-FUNDC1 binding region in second cytosolic domain especially amino acid (AA) 109-118 (fig 2L). Further, when mutated AA at this region one by one, N118 was found to be the crucial AA for the interaction (fig 2M and fig 2N).**

8. Figure 5 showed that ULK1 is important for mitophagy induced by hypoxia or FCCP. However, as ULK1 is also essential for autophagy in general, it is inappropriate to conclude here that ULK1 has a specific role in mitophagy regulation. Also, the protein level change of TIM23, TOM20 or VDAC1 is not so significant in these experiments.
**Answer: Thanks for the suggestion. We modified our conclusion from "ULK1 is critical for hypoxia-, FCCP-, or FUNDC1-induced mitophagy" to "ULK1 is required for hypoxia-, FCCP-, or FUNDC1-induced mitophagy". To confirm the conclusion, we repeated the experiments and found that the autophagic degradation of mitochondrial proteins is significant (fig 3). To show more clearly, we quantified the representative bands and statistics are shown (fig S8). We also found that it is active ULK1 (kinase activity is normal) but not the kinase dead form of ULK1 that plays an important role in mitophagy (fig3D and E).**

9. In Figure 5E, the FUNDC1-myc expression induced a significant degradation of TIM23 under ULK1 (-/-) conditions; what is the explanation for this contradictory result (notice that TOM20 degradation is completely blocked under the same conditions)?
**Answer: This may be due to lacking of quantification and statistics. To draw a solid conclusion, we repeated the experiments twice again and quantified the representative bands and found that FUNDC1 could potentially induce autophagic degradation of VDAC1, TIM23 and TOM20 which is largely impaired in ULK (-/-) cells (fig 3H). The statistics are shown in**

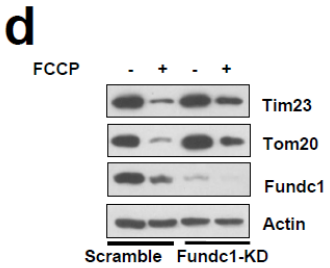
**fig S8. We used an alternative method to strengthen the conclusion. We measured the citrate synthase activity under this condition and found that FUNDC1 (S17D) indeed induced significant reduction of mitochondrial activity (fig 3I).**

10. In Figure 6A, the protein level change of TOM20 is so insignificant that it hardly reflects a mitophagy activity change. Quantification of the intensity of the band may help the authors achieve a better conclusion.
**Answer: Thanks for the suggestion. As we quantified the band of TOM20 in the previous blot, the protein level of TOM20 reduced significantly compared with the first line, which reflects a significant mitophagy activity change. To confirm the results, we repeated the experiments twice again and added an additional marker VDAC and found that the mitophagy is significant. Quantification and statistics are shown in fig 3F and S8.**

11. Was the anti-FUNDC1 (Ser17) antibody able to detect FUNDC1-S17D?
**Answer: Yes it is (fig S3).**

12. Where is the anti-VDAC1 blot in Figure 7A, as it is shown in Figure 7B?
**Answer: Sorry for the missing VDAC1. We added the VDAC1 band to the new fig 4A.**

13. Overall, the authors make no distinction between hypoxia- or FCCP-induced mitophagy. However, the previous report (Liu et al, 2012) pointed out that FUNDC1 is primarily involved in hypoxia-induced mitophagy, but involved to a significantly lesser extent in FCCP-induced mitophagy. How do the authors reconcile this result within their current study?
**Answer: Actually, in our previous paper, knockdown of Fundc1 inhibits at least 50% loss of mitochondrial proteins compared to scrambled control in FCCP-induced mitophagy in HeLa cells (figS3D in NCB paper, 2012, also see the raw data from that paper below). We thus concluded that FUNDC1 is partially involved in FCCP-induced mitophagy in HeLa cells (line4, pp.180, NCB, 2012). In this manuscript, we found that ULK1 is associated with FUNDC1 under hypoxia or FCCP condition. ULK1 depletion alone sufficiently suppresses the mitophagy induced by both conditions and reintroducing ULK1 and FUNDC1 into cells that lacks ULK1 and FUNDC1 expression promotes more potent mitophagy (figs 3 and 4). In this manuscript, we want to mainly address the function of ULK1 in selective mitophagy and to prove that FUNDC1 is its novel substrate in this process; we want to investigate the coordinated role of both ULK1 and FUNDC1, and to show that FUNDC1 may strengthen the activity of ULK1 in mitophagy induced by hypoxia or FCCP, and we do not want to exaggerate the function of FUNDC1 in mitophagy induced by both conditions. We wish the revised manuscript would have fully addressed the concerns of this referee.**




Referee #2:

Wu W et al. address the question of how dysfunctional mitochondria are sensed by the phagosome and the early mitophagy regulator ULK1, respectively. They show that ULK1 is upregulted and translocates to damaged mitochondria in response to hypoxia or FCCP. Mass spectrometric analysis of ULK1-coprecipitating proteins identifies the mitochondrial membrane protein FUNDC1, a mitophagy receptor for hypoxia-induced mitophagy, as a binding partner of ULK1. These results were confirmed by Co-IPs after transient transfection and with endogenous proteins. Based on some unpublished data of the authors, they generate an anti-FUNDC1 (Ser-17)-specific antibody as Ser-17 was found to be a potential hypoxia-induced phosphorylation site. This antibody was used to show

that FUNDC1 phosphorylation is dependent on the presence of ULK1 in cells and in vitro. The authors also attempt to define the region in FUNDC1 mediating the interaction with ULK1. Using siRNA-mediated knockdown of FUNDC1 they want to stress that FUNDC1 is required for translocation of ULK1 to damaged mitochondria. This was demonstrated by quantit. immunofluorescence as well as subcellular fractionation. The authors then analyse the functional importance of ULK1 for hypoxia-induced mitophagy. Using ULK1-/- MEFs reconstituted with either wt or kinase-dead ULK1 they demonstrate that ULK1 kinase activity is required for efficient mitophagy as assessed by autophagic degradation of mitochondrial proteins including TIM23, TOM20 and VDAC1. In order to elucidate the role of FUNDC1 Ser-17 phosphorylation in this process a phosphomimetic mutant FUNDC1 S17D was constructed. This mutant was analysed in ULK1-/- cells and was able to restore mitophagy while wt or S17A failed to do so. The authors also present data that S17-phosphorylation increases the association of FUNDC1 with LC3.

Collectively, the presented data suggest that ULK1 is able to associated with and phosphorylate FUNDC1 in conditions of hypoxia or FCCP which might play a role in autophagic clearance of damaged mitochondria. In addition to some technical issues (see comments below) the main criticism is that the authors fail to solve the crucial question, namely how ULK1 discriminates between damaged and healthy mitochondria. What exactly triggers ULK1 translocation to mitochondria in response to hypoxia or FCCP? Also, the in vivo significance of the observed ULK1/FUNDC1- interaction remains an open issue. In particular, the data showing that ULK1 plays a critical role in hypoxia-induced mitophagy was not convincing. Without solving these issues the current manuscript is preliminary and lacks sufficient novelty. I cannot recommend it for publication in EMBO J.

**Answer: The issues raised by this referee are very interesting and indeed they are really good scientific questions need to be investigated in our future work. Fundc1 knockout mouse has not been published yet and it needs a long time to get the KO mouse (is now being constructed by our collaborators), which makes *in vivo* significance of ULK1-FUNDC1 interaction hard to be addressed here. In this manuscript, we want to mainly address the function of ULK1 in selective mitophagy and to prove that FUNDC1 is its novel substrate in this process; we want to investigate the coordinated role of both ULK1 and FUNDC1, and to show that FUNDC1 may strengthen the activity of ULK1 in mitophagy induced by hypoxia or FCCP. So, how ULK1 itself is activated is another complex question and it seems to be beyond the scope in the current study. We believe that FUNDC1 is probably mediating the ULK1 translocation since knockdown of FUNDC1 or transfecting ULK1 binding mutant (FUNDC1 N118A) to FUNDC1 KD cells prevents ULK1 translocation and mitophagy. At present, we have no idea about what triggers the ULK1 translocation. We will test the upstream stimulator of ULK1 in our follow-up work.**

Comments:

1. ULK1 is known to trigger starvation-induced autophagy. Does starvation also lead to mitochondrial translocation and FUNDC1 phosphorylation?

**Answer: Thanks for the suggestion. We studied this issue under the starvation condition and found that starvation does not lead to ULK1 translocation and FUNDC1 phosphorylation (figure S1 and S5).**

2. Fig. 2F: The authors might want to show the input.

**Answer: We have shown the input in the new figure 2. The experimental procedure is: We combined the immunoprecipitated myc-ULK1 from cell lysates with *E.coli* purified GST, GST-FUNDC1 or GST-FUNDC1 proteins to do the *in vitro* kinase assay. Then, we blot the gel with anti-MYC, FUNDC1, GST, ser-17 phosphorylation antibodies, respectively.**

3. The authors wish to determine the ULK1-binding region on FUNDC1. However, full length FUNDC1 is a protein of 155 aa and the "binding region" defined by the authors spans aa 50-139. To me this does not sound very defined. Moreover, what is the sense of defining a binding region if this information is not used for further experiments? Indeed, a FUNDC1 (point) mutant that is deficient in ULK1-binding would be very helpful for functional analysis in cells.

**Answer: We made a series of deletions and mutations to define the minimal binding region and found that N-118 which localizes at the cytosolic loop domain plays a crucial role in the FUNDC1-ULK1 interaction (fig 2I-N). Mutation of N 118 to A abolishes their interaction.**

4. Fig. 4: The authors quantified the effect of FUNDC1 knockdown on ULK1 recruitment to mitochondria in response to hypoxia/FCCP. The number of ULK1-positive mitochondria obviously decreases (from around 50 % to around 16 %). However, given the very efficient knockdown of FUNDC1 it is surprising that a significant amount of mitochondria is still positive for ULK1. In addition, the most striking effect of FUNDC1 knockdown seems to be that ULK1 is not efficiently upregulated in response to hypoxia/FCCP (see Fig. 4A). Rather than the lack of FUNDC1 this low expression level might explain the significant decrease of ULK1-positive mitochondria.
**Answer: We think the expression of ULK1 in FUNDC1 KD cells is obvious in response to hypoxia/FCCP (fig S6C and D), please compare quantification of the ULK1 expression in NC (PNS+Cyto) and SI (PNS+Cyto)). The amount of ULK1 in the NC lysate is lower than in the SI lysate, nevertheless, ULK1 in NC still translocates). Fig. S6A also shows considerable upregulation of ULK1 and significant ULK1-mitochondrial colocalization in response to hypoxia/FCCP compared to control. The puncta is scattered in the cytoplasm, so it looks not efficiently upregulated. We also identified the binding-deficient mutant and showed that FUNDC1 is indeed important for the ULK1 association with mitochondria and mitophagy (fig 2I-P).**

5. In order to corroborate the notion that binding of FUNDC1 to ULK1 and Ser-17 phosphorylation is crucial for mitophagy the authors should generate binding-deficient mutants instead of knocking down the whole protein. As both proteins have established functions in mitophagy it is not surprising that their removal causes a mitophagy defect. It rather needs to be shown that specific disruption of that particular interaction is responsible for the observed defect.
**Answer: Thanks for the suggestion. This is a further question of point 3. We used FUNDC1 N118A as the binding-deficient mutant and found that this mutant significantly inhibits mitophagy in FUNDC1 KD cells, compared to (FUNDC1 K110A), which still has ULK1 binding ability (fig 2I-P).**

6. Fig. 5A/B: The effect of ULK1 on hypoxia-induced degradation of mitochondrial proteins is rather little. The quantification and a statistical analysis of at least three independent experiments are required for solid conclusions.
**Answer: This is due to no quantification. To confirm the results, we repeated the all the experiments and showed the quantification and statistics in the new fig 3 and fig S8.**

7. Fig. 5E: Tim23 degradation does not seem to be impaired in ULK-/- MEFs. Quantification and statistical analysis are required.
**Answer: Thanks for the comments. We added the quantification and statistics in the new fig 3 and fig S8.**

8. Fig. 7 A and B: Why is there a signal for endogenous ULK1 in the lanes with ULK1-/- MEFs??
**Answer: We are sorry for our unclear mark in our original figure. Actually, the ULK1 lanes which were shown in the figure are exogenous transfected HA-ULK1. (Fig 4A and B).**

9. Fig. 7B: Only Tim23 levels are decreasing in ULK1+/+ MEFs in response to FCCP.
**Answer: This is due to no quantification. We quantified the band and found that other mitochondrial markers also decrease significantly. Please note that the bands in lane 2 (TIM23, TOM20 and VDAC) is narrower than lane 1. So, the actual protein level is much less in response to FCCP when we quantified the bands with imageJ (fig 4B and S12).**

10. In general: the data showing that ULK1 critically participates in hypoxia-induced mitophagy are not consistent.
**Answer: We performed many critical experiments and weakened some conclusions to address concerns of this referee and we wish it would satisfy the referee. In this manuscript, we found that ULK1 is elevated and associated with its novel substrate FUNDC1 under hypoxia or FCCP induced selective autophagy (figs 1 and 2). ULK1 depletion alone sufficiently suppresses the mitophagy induced by either hypoxia or FCCP and reintroducing ULK1 and FUNDC1 into cells that lacks ULK1 and FUNDC1 expression promotes more potent mitophagy (figs 3 and 4). Point mutant FUNDC1 (N118A) prevents ULK1-FUNDC1 binding, colocalization and mitophagy. We want to investigate the function of ULK1 in selective mitophagy, the**

**coordinated role of both ULK1 and FUNDC1, and to show that FUNDC1 may strengthen the activity of ULK1 in mitophagy induced by hypoxia or FCCP.**

Referee #3:

In the manuscript by Wu et al., the authors add more detail to the mechanism of FUNDC1-mediated mitophagy following a previous publication. They show that ULK1 is required for the process and that it binds to and phosphorylates FUNDC1 to drive LC3 association and mitophagy. I am enthusiastic about this manuscript as it provides insight into how a key player in general autophagy (ULK) functions in targeting specific components. The search for ULK substrates is of great interest in the autophagy field and although substrates have already been identified (VPS34 complex), this is the first one to function in a specific form of autophagy. I do however have some concerns, such as the lack of quantitation and statistical analysis of the western blot/IF data as well as additional experiments to address the differential phosphorylation by Src/ULK1.

**Answer: Thanks for the appreciation of our work and the invaluable comments. We added additional experiments and wish our answers would fully satisfy the referee.**

Main points:
1) Recent work from the Yoshimori lab has suggested that autophagosomes form at ER-mitochondria contact sites (Hamasaki et al., Nature 2013). It is therefore possible that FUNDC1 is recruiting ULK1 to this area for general autophagy. I know the previous publication showed that that KD of FUNDC1 did not alter LC3 lipidation, but in light of the role of ULK1 here, I think it important to show that depletion of FUNDC1 does not block ULK1 puncta formation under starvation-induced autophagy.
**Answer: This is a very good advice. We performed the experiment as this reviewer pointed out and found that depletion of FUNDC1 does not block ULK1 puncta formation under starvation-induced autophagy (fig S1) and starvation also does not trigger the Ser-17 phosphorylation at FUNDC1 (fig S5).**

2) In the previous FUNDC1 publication (Lui et al., 2012), the authors claimed that FUNDC1 depletion blocked hypoxia-induced mitophagy, but only partially affected that induced by FCCP and had no effect of loss of mitochondria observed by long term starvation. This lead to the conclusion that FUNDC1 was specific for hypoxia-induced mitophagy. In the current manuscript the authors are now proposing a role for FUNDC1 in FCCP mitophagy - can they explain this discrepancy? Also, given the previous data, does rescue with the FUNDC1 S17A mutant alter starvation mitophagy?
**Answer: Actually, in our previous paper, knockdown of FUNDC1 inhibits at least 50% loss of mitochondrial proteins in FCCP-induced mitophagy in HeLa cells (figS3D in NCB paper, 2012). We thus concluded that FUNDC1 is partially involved in FCCP-induced mitophagy in HeLa cells (line4, pp.180, NCB, 2012). In this manuscript, we found that ULK1 is associated with its new substrate FUNDC1 under hypoxia or FCCP conditions. ULK1 depletion alone sufficiently suppresses the mitophagy induced by both conditions and reintroducing ULK1 and FUNDC1 into cells that lacks ULK1 and FUNDC1 expression promotes more potent mitophagy (figs 3 and 4). We want to mainly address the function of ULK1 in selective mitophagy, the coordinated role of both ULK1 and FUNDC1, and to show that FUNDC1 may strengthen the activity of ULK1 in mitophagy induced by hypoxia or FCCP and we do not want to exaggerate the function of FUNDC1 in mitophagy induced by both conditions. Since knockdown of FUNDC1 has no effect on starvation-induced loss of mitochondria as shown in our previous paper, since FUNDC1-S17 is not phosphorylated by ULK1, and since ULK1 does not translocate to mitochondria in starvation condition (figs S1 and S5), we think FUNDC1 does not affect starvation mitophagy.**

3) In Fig 2 it is important to indicate the relative ratios of input lysate to IP, in order to know the efficiency of IP - on first look it appears that there has been no enrichment during the IP for the majority of experiments making it hard to assess the relevance of the data.
**Answer: We indicated the relative ratios of input lysate to IP (1/20) in the revised manuscript (fig 2A and 2B). To confirm the results, we performed a series of IP experiments for different FUNDC1 truncations and some of them showed strong binding (fig 2I-N). Also, we re-**

**performed the endogenous IP for ULK1 and FUNDC1 in mitophagy induced by hypoxia or FCCP. ULK1 can immunoprecipitate FUNDC1, compared to IgG or untreated control (fig 2C).**

4) All the FUNDC1 phosphorylation data in Fig 2 would really benefit from quantitation. The wt vs kinase-dead ULK data is not as strong as would be expected - especially in panel D if you normalize to total FUNDC1. Are the authors sure that their K46N ULK mutant is kinase dead as there still appears to be significant phosphorylation in panel E and F? It would help if the authors blotted for a previously identified ULK1 site to confirm this, such as phosphoS318-ATG13 (Abnova).
**Answer: Thanks for the suggestion. The phosphorylation signals of ULK1 WT and ULK1 K46N are quite different when we quantified the bands that normalized to total FUNDC1 (fig 2D, E). This is because ULK1 K46N still has minor kinase activity as it weakly phosphorylates its substrate ATG13 (fig S4).**

5) Based on the topology of FUNDC1, it appears likely that ULK would interact with one of the cytosolic loops. In Fig. 3, the authors show that it does not interact with the first cytosolic domain (1-50), suggesting that the interaction occurs between residues 96-139. Why did the authors not try this construct? The 96-155 construct does not show any interaction, but based on the IF staining it looks to form aggregates and therefore might not interact because of this. Do these constructs localize to mitochondria?
**Answer: This is a very good advice. We addressed the concern by construct a serials of new FUNDC1 mutants and deletions (fig 2I-N). We found that FUNDC1 lost its mitochondrial localization if the first and second transmembrane domains are simultaneously deleted (Because 1-139, 69-138, 50-71/97-155 localized to mitochondria while 96-155 does not) (fig 2I and S7). We also found that mitochondrial localization of FUNDC1 is necessary but not sufficient for its interaction with ULK1 because 1-96 has mitochondrial localization but does not interact with ULK1 (fig 2I-N and S7). Besides, we found that N118 which localizes in the cytosolic loop plays a crucial role in the FUNDC1-ULK1 interaction (fig 2). FUNDC1 N118A abolishes their binding.**

6) The data in Figure 5 require quantitation. With the exception of VDAC1 (misspelt as VADC1 in panels A and C) and TOM20 there appears to be little turnover of protein. It is of concern because TOM20 and VDAC1 have been proposed to be turned over by the proteasome prior to mitophagy induced by depolarization (Yoshii et al., JBC 2011 and Chan et al., Hum Mol Genet 2011). It looks like there is some rescue by the addition of bafilomycin, but is hard to tell as there is no quantitation. Do the authors know that proteasomal inhibition does not rescue TOM20/VDAC levels? I also think it important to include an untreated sample plus bafilomycin for 24h to show that the effects are specific to induced mitophagy vs. basal mitophagy.
**Answer: Thanks for the comments. To confirm our results, we repeated all the WB experiments and quantified all the bands in the new representative figures in the new manuscript. and we believe that the turnover of mitochondrial proteins is significant which can be dramatically reversed by lysosome inhibitor bafilomycin (fig 3). We also tested the TOM20/VDAC levels in mitophagy induced by hypoxia or FCCP with or without lysosome inhibitor bafilomycin or proteasome inhibitor MG132. We found that TOM20/VDAC/TIM23 degradation induced by hypoxia can be reversed by BAF but not by MG132, while turnover of TOM20/VDAC/TIM23 induced by FCCP can be reversed by BAF but to a lesser extent by MG132, indicating that besides mitophagy, proteasome degradation of mitochondrial proteins is also involved in FCCP-induced mitochondrial turnover (fig S8 and S10). There is no obvious protein level change between untreated sample and untreated sample plus bafilomycin.**

7) Also in Fig 5E, FUNDC1 overexpression is inducing mitophagy in ULK1 null MEFs, despite what the authors claim (quantitation would confirm this). It is clearly inducing autophagy as seen by LC3-II flux. This is contrary to the authors arguments - do they have an explanation?
**Answer: It is also reportedly proposed that in some cases, LC3-II localizes to protein aggregates (not incorporated to autophagosome membrane) although LC3 lipidation is normally processed (Kuma A, *et al*. Autophagy, 2007). So, the effective way is to test the level of autophagy substrate p62. We compared the p62 level in both conditions. Although FUNDC1 WT promotes LC3 lipidation, it does not trigger p62 degradation and mitophagy in ULK1 null MEFs compared to the phospho-mimetic FUNDC1 (S17D) (fig 3H). This is also confirmed by**

**results in fig 3G where mitochondria do not colocalize with LC3 puncta in ULK1 null MEFs which are transfected by FUDNC1 WT.**

8) The data in Fig 6 require quantitation. There appears to be differential degradation of mitochondrial markers, for example in panel A there is loss of TIM23, but relatively very little loss of TOM20. In panel B, the situation is reversed. Why the inconsistencies? I think it important for the authors to use an additional method to quantitate mitochondrial abundance under these conditions (+/- bafilomycin), such as biochemical measurement of citrate synthase activity (this is commonly used and there are many published protocols).
**Answer: To confirm the results, we repeated the experiments and quantified the indicated WB bands. Although there is slightly differential degradation of TIM23 and TOM20, they are degraded significantly (figure 3 and S8). We also added the suggested method (biochemical measurement of citrate synthase activity to quantitate mitochondrial abundance and found that the citrate synthase activity in figure 3I is consistent with the WB result in Figure 3H.**

9) Based on the previously published data on FUNDC1 Y18 phosphorylation, I think the authors have missed some important experiments that would be very helpful in explaining the mechanism of ULK recruitment and mitophagy. These are adjacent sites and one is inhibitory for mitophagy (Y18), while the other stimulatory (S17). What is their relationship - are they mutually exclusive? Is the Y18 phosphorylated in the S17D mutant, or hyperphosphorylated in the S17A mutant? Does overexpression of Src block S17 phosphorylation/S17D-induced mitophagy/recruitment of ULK to mitochondria? While I don't think it necessary to answer all of these questions in detail, some insight would be very useful for mechanism and should be relatively easy to carry out since the lab has all the reagents.
**Answer: This is a very good suggestion. We performed the experiments and found that Y18 and S17 are mutually exclusive. The Y18 is dephosphorylated in the S17D mutant, and is hyperphosphorylated in the S17A mutant (fig S3). Overexpression of Src largely suppresses S17 phosphorylation and the ULK1 and mitochondria colocalization (fig S11).**

10) Quantitation for figure 7 is needed, especially for the EM. What are the dark, electron-dense structures and do they have any relevance to mitophagy?
**Answer: We quantified both the WB and the EM for the figure 7 (now fig 4A, B, S13A, B, S12 and fig 4D, E). Only the mitochondria-containing autophagic structures in the EM were marked.**

Minor points:
1) How enriched are the subcellular fractions in mitochondria? If they are not pure then it is misleading to represent them solely as the mitochondrial fraction. The IF co-localization of ULK and TIM23 is only partial, so it would be useful to know if other membrane markers are present in the mitochondrial fraction, such as lysosomes (eg. LAMP1) or endosomes (eg EEA1) as these could be responsible for the ULK levels in the western blot. Perhaps "Mitochondrial-enriched" as a fraction label would be more appropriate if there are contaminants?
**Answer: We followed a very sophisticated and well-established mitochondrial purification protocol. We added two other membrane markers LAMP1 for lysosome and EEA1 for endosomes which were not in the mitochondrial fraction, suggesting that the mitochondria fraction is pure (figs 1F and 1G).**

2) The FUNDC1 exposure in panel 2A is rather low, making the band hardly visible - do the authors have a longer exposure.
**Answer: We think that the referee refers to fig 7A (new fig 4A). We changed the light contrast of the panel to make it look clearer. We are very sorry for that we do not have a longer exposure.**

1st Editorial Decision 13 February 2014

Thank you for your patience while your study has been under peer-review. We have now received the enclosed reports from the referees that were asked to assess it. Referee 1 from your previous

submission to The EMBO Journal was not available at this time, so I secured an additional referee (#3). Former referee 3 is now #1 (former referee 2 remained #2).

As you will see, scientifically the study only needs to address two minor concerns from referee #2 before acceptance: analysis of Ser17 phosphorylation in FUNDC1 (N118A) and replacement of some panels of figure 2P.

In addition, all referees allude to the fact the current text is unclear and does not highlight the novelty or the study or previous literature in sufficient detail. I will increase our length limit to 31,000 characters so that you can better address this issue and some other ones that I detail below. In addition, I agree with referee 2 that panels I-L in figure 2 could be moved to the supplement now, to tidy up the figure and enlarge the IF a bit.

From an editorial standpoint, a few things need addressing, as follows:

- As a routine procedure, we now request the publication of original source data for the main figures -particularly for electrophoretic gels and blots, and optionally also for graphs. This makes primary data more accessible and transparent to the reader. Please provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all gels used in the figures and, if desired, an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

- The materials and methods section is rather succinct. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information may be included as Supplementary Material. Please also move the subheading on statistical analysis of the data to the main text.

- There are a few missing details in the figure legends regarding the analyses performed. Please ensure that all relevant figure legends (including those of supplementary figures) have information regarding what is represented by the bar (mean, median?) and error bars, as well as the number of independent experiments analyzed, statistical test used and values considered significant. When the same information applies to several panels in one figure, it can be included at the end of the figure legend, indicating the panels it applies to. This will help in saving space for the main text.

- It is unclear to me what the red lines below some of the blots represent. Please note that if the data depicted comes from different gels, the splices need to be clearly indicated within the gel itself.

In addition, in the LC3 I-II blot in figure 3D, the leftmost lane (control) has a rather peculiar pattern (please compare to figs 3E and 3F, for example). Perhaps you would have a more representative replicate of the experiment?

Once you have made these minor revisions, please upload the final version of your study and all associated files through our online system, accompanied by a point by point response to all referee and editorial concerns.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The manuscript by Wu et al., is a resubmission of a version previously reviewed at the EMBO Journal. The authors have addressed all my previous concerns and the work now appears to be much stronger scientifically and is certainly worthy of publication - I am just not sure whether EMBO Reports is the right place. Clearly a lot of work has gone into the manuscript, but given the short-format type of paper required by the journal, the authors have had to condense the paper

dramatically. This is obvious when looking at the figures - Figure 2 has 16 panels and the rest are not far behind! There are also 13 supplemental figures too. The text also needs expanding to put the findings into context with what is already known and to drive home the novelty of the work. The end result is that the manuscript is difficult to digest and the key points do not really come across. The manuscript just reads like a brief description of all the data. I do not think the short-format does the manuscript justice. Potentially the authors could take out a lot of data, such as the FCCP data and just focus on the hypoxia-driven mitophagy, but my opinion is that the manuscript would be better off containing the majority of data and published as "long-format" paper.

Referee #2:

The authors have significantly improved the quality of their manuscript. The requested quantifications and statistics have been included. Importantly, they have provided the ULK1 binding-deficient FUNDC1 mutant and shown that this mutant is unable to support mitophagy. While the major concerns have been addressed there remain some minor issues:

1. The authors should show that the ULK1 binding deficient mutant (FUNDC1 N118A) is not phosphorylated on S17 in cells. In addition it should be also included in the experiment shown in Fig. 4 A and B.

2. On page 4/5 (paragraph on „Kinase active form of ULK1 is required for hypoxia-, FCCP-, or FUNDC1-induced Mitophagy") the text flow is not logic anymore because of the insertion of additional text.

1. Fig. 2 I-L: Since the authors present new data on the point mutant of FUNDC1, these figures could be moved to the supplement. The IF image can be enlarged instead as it is very small in the current version.

2. New Fig. 2P: The authors should show untreated cells. Moreover, the panel showing FUNDC1 K110A expressing cells after FCCP treatment is not convincing. Co-localization of FUNDC1 K110A, ULK1 and Mito does not differ significantly from the panel below showing FUNDC1 N118A. The authors might want to select cells with higher expression levels to clarify this. Taken together, the revised manuscript can be considered for publication.

Answers to referee #1

The main concerns of this referee were related to the obvious technical weakness of many experiments and to the clear distinction between hypoxia and FCCP-induced mitophagy. In the revised version the authors have provided a quantification of most experiments and added several new data that indeed support their conclusions. Also the issue about hypoxia vs. FCCP-induced mitophagy was convincingly discussed.

Referee #3:

In the present study Wu et al. provide further evidence for the involvement of the mitochondrial protein FUNDC1 in mitophagy. Specifically, the authors provide evidence that FUNDC1 serves as a substrate for ULK1, Atg1 mammalian ortholog. The authors identified Ser 17 in FUNDC1 as the main target for ULK1 and also found the interaction region in FUNDC1 essential for the interaction with the kinase. Finally, functional evidence is provided to link between FUNDC1 phosphorylation and mitophagy.

This is both interesting and important study. The manuscript was initially reviewed by 3 referees

who's comments help improve the manuscript significantly. I find the authors' response to the referees comments (mainly the experimental part) satisfactory, leading to a study that meets the scientific merit of EMBO Reports. The authors however still need to improve and clarify the text.

1st Revision - authors' response 18 February 2014

Editor's COMMENTS

1 As a routine procedure, we now request the publication of original source data for the main figures -particularly for electrophoretic gels and blots, and optionally also for graphs. This makes primary data more accessible and transparent to the reader. Please provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all gels used in the figures and, if desired, an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

**Answer**: We demonstrated the original source data for the main figures. The uncropped and unprocessed scans are shown in a new PDF file, which will be uploaded together with other files.

2 The materials and methods section is rather succinct. Please note that basic Materials and Methods required for understanding the experiments performed must remain in the main text, although additional detailed information may be included as Supplementary Material. Please also move the subheading on statistical analysis of the data to the main text.

**Answer**: Considering to the limitation of character number, we just added few main experiment details to the materials and methods section. Besides, we also move the subheading on statistical analysis of the main data to the main text in the new version.

3 There are a few missing details in the figure legends regarding the analyses performed. Please ensure that all relevant figure legends (including those of supplementary figures) have information regarding what is represented by the bar (mean, median?) and error bars, as well as the number of independent experiments analyzed, statistical test used and values considered significant. When the same information applies to several panels in one figure, it can be included at the end of the figure legend, indicating the panels it applies to. This will help in saving space for the main text.

**Answer**: We added the missing details in the figure legends regarding the analyses performed. As shown in the new text, we have noted the information of the bar and error bars in the legends.

4 It is unclear to me what the red lines below some of the blots represent. Please note that if the data depicted comes from different gels, the splices need to be clearly indicated within the gel itself.

In addition, in the LC3 I-II blot in figure 3D, the leftmost lane (control) has a rather peculiar pattern (please compare to figs 3E and 3F, for example). Perhaps you would have a more representative replicate of the experiment?
**Answer**: The red lines are just used to make the statistical numbers clearly, and these are not any spliced panels in these figures. In the new version, we deleted the red lines below the blots.
We have replaced the panel of LC3 in the new version with a long exposure panel.

5 As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please read the edited version carefully and let me know if you do not agree with any of the changes.
**Answer**: We like the edited title and abstract of the manuscript by the editor. Only need to change the words in the abstract “constitutively active” to ”kinase active”, since there is no constitutive active ULK1 used.

6. Lastly, every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text -I have added my proposal for this text below- as well as 2-4 one sentence bullet points that summarise the paper. These should be complementary to the abstract -i.e. not repeat the same text. This is a good place to include, as appropriate, key acronyms and quantitative information. Could you supply a 211 pixels wide by 157 pixels high graphic outlining the main message of the study? Perhaps a higher resolution version the scheme in Fig 4F would be nice. Do let me know if you would like to modify the synopsis text:
This study shows that ULK1 translocates to damaged mitochondria and phosphorylates FUNDC1, which is crucial for organelle elimination through mitophagy. ULK1-induced phosphorylation enhances FUNDC1 interaction with LC3.

- hypoxia and mitochondrial uncouplers upregulate ULK1 and induce its translocation to damaged mitochondria
- ULK1 recruitment to mitochondria is regulated by its binding to FUNDC1
- at mitochondria, ULK1 directly phosphorylates FUNDC1 at serine 17, which is necessary for FUNDC1-LC3 binding and colocalization
- serine 17 phosphorylation is required for mitophagy in response to hypoxia and FCCP
**Answer**: The synopses summarized our work briefly and correctly. In addition, we will supply the higher resolution of outlining graph to make the synopsis more accessible.

Referee #1
The manuscript by Wu et al., is a resubmission of a version previously reviewed at the EMBO Journal. The authors have addressed all my previous concerns and the work now appears to be much stronger scientifically and is certainly worthy of publication - I am just not sure whether EMBO

Reports is the right place. Clearly a lot of work has gone into the manuscript, but given the short-format type of paper required by the journal, the authors have had to condense the paper dramatically. This is obvious when looking at the figures - Figure 2 has 16 panels and the rest are not far behind! There are also 13 supplemental figures too. The text also needs expanding to put the findings into context with what is already known and to drive home the novelty of the work. The end result is that the manuscript is difficult to digest and the key points do not really come across. The manuscript just reads like a brief description of all the data. I do not think the short-format does the manuscript
justice. Potentially the authors could take out a lot of data, such as the FCCP data and just focus on the hypoxia-driven mitophagy, but my opinion is that the manuscript would be better off containing the majority of data and published as "long-format" paper.
**Answer**: Thank you for your appreciation and advices. The editor increased our character limitation; this gave us more space to elaborate our discovery. In the new version, we added more details of the results and discussed the work deeply. At the same time, we reorganized the figures to smooth the results and put some into the supplement. The revised manuscript would be more accessible and easy to digest.

Referee #2
The authors have significantly improved the quality of their manuscript. The requested quantifications and statistics have been included. Importantly, they have provided the ULK1 binding-deficient FUNDC1 mutant and shown that this mutant is unable to support mitophagy. While the major concerns have been addressed there remain some minor issues:
1. The authors should show that the ULK1 binding deficient mutant (FUNDC1 N118A) is not phosphorylated on S17 in cells. In addition it should be also included in the experiment shown in Fig. 4 A and B.
**Answer**: Thank you for this advice. We have analyzed the phosphorylated on S17 of FUNDC1 in FUNCDC1 N118A transfected cells. The new panel was added to figure2k in the new version. In the Fig 4A and B, we also added the new bands of the phosphorylation of S17.
2. On page 4/5 (paragraph on ?Kinase active form of ULK1 is required for hypoxia-, FCCP-, or FUNDC1-induced Mitophagy") the text flow is not logic anymore because of the insertion of additional text.
**Answer**: We have revised the text. In this new manuscript, the insertion of additional text moved to afterwards. This new paragraph would be logic and fluent now.

3. Fig. 2 I-L: Since the authors present new data on the point mutant of FUNDC1, these figures could be moved to the supplement. The IF image can be enlarged instead as it is very small in the current version.
**Answer**: We have moved these figures to the supplement and enlarged the IF image in the revised version.

4. New Fig. 2P: The authors should show untreated cells. Moreover, the panel showing FUNDC1 K110A expressing cells after FCCP treatment is not convincing. Co-localization of FUNDC1 K110A, ULK1 and Mito does not differ significantly from the panel below showing FUNDC1 N118A. The authors might want to select cells with higher expression levels to clarify this. Taken together, the revised manuscript can be considered for publication

**Answer**: We added the IF of untreated cells as the control. Co-localization of FUNDC1 K110A, ULK1 and Mito after FCCP treatment was replaced in the new fig 2, where the colocalizations are prominent.

Referee #3:

In the present study Wu et al. provide further evidence for the involvement of the mitochondrial protein FUNDC1 in mitophagy. Specifically, the authors provide evidence that FUNDC1 serves as a substrate for ULK1, Atg1 mammalian ortholog. The authors identified Ser 17 in FUNDC1 as the main target for ULK1 and also found the interaction region in FUNDC1 essential for the interaction with the kinase. Finally, functional evidence is provided to link between FUNDC1 phosphorylation and mitophagy.

This is both interesting and important study. The manuscript was initially reviewed by 3 referees who's comments help improve the manuscript significantly. I find the authors' response to the referees comments (mainly the experimental part) satisfactory, leading to a study that meets the scientific merit of EMBO Reports. The authors however still need to improve and clarify the text.

**Answer:** We thank this reviewer for the positive appraisal of our work. We are sorry for that we did not clarify our text because of the space limitations. In the revised manuscript, we improved the text and discussed the work of others appropriately.

2nd Editorial Decision 19 February 2014

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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication.