EMBO reports

Manuscript EMBOR-2013-37156

Casein kinase 2 is essential for mitophagy

Tomotake Kanki, Yusuke Kurihara, Xiulian Jin, Tadahiro Goda, Yusuke Ono, Masamune Aihara, Yuko Hirota, Tetsu Saigusa, Yoshimasa Aoki, Takeshi Uchiumi and Dongchon Kang

Corresponding author: Tomotake Kanki, Niigata University Graduate School of Medical and Dental Sciences

Review timeline:

Submission date:	05 February 2013
Editorial Decision:	19 February 2013
Revision received:	13 June 2013
Editorial Decision:	28 June 2013
Revision received:	07 July 2013
Accepted:	11 July 2013

Editor: Nonia Pariente

Transaction Report:

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

1st Editorial Decision 19 February 2013

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for us, they raise a number of issues that need to be addressed in a round of revision to strengthen the study.

All referees provide constructive experimental suggestions that would improve the he work and should be taken into account. I appreciate that referee 3's first major concern regarding the lack of Atg32 phosphorylation when mitophagy is not induced is a further reaching issue. I would encourage you to try to address it -as this would undoubtedly raise the significance of the work- however, publication in EMBO reports would not depend on elucidating this point. On the other hand, please note that all the other referee comments should be taken on board. If they can be adequately addressed, we would be happy to accept your manuscript for publication. It is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless

previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

We also welcome the submission of cover suggestions or motifs that might 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. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

****
Note:

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. 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.

You are able to opt out of this by letting the editorial office know (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."

****

REFEREE REPORTS:

Referee #1:

Summary
1) Yes, the manuscript by Kanki et al convincingly shows a direct role of CK2 in mitophagy by phosphorylating Atg32.

2) This manuscript is of significance.

3) This is of interest for the molecular biology community as it describes the first kinase directly phosphorylating an autophagy receptor in yeast. Unraveling the mechanism of organelle turnover is fundamental to our understanding of the biology of cells.

4) Yes

One additional experiment would really improve the study. It can be easily tested whether the interaction of Atg32 with Atg11 and Atg8 is dependent upon CK2 activity. Should easily be done in 1-2 month.

The manuscripts needs correcting for English grammar in some places.

Report
The manuscript by Kanki et al convincingly shows a direct role of CK2 in mitophagy by phosphorylating Atg32. CK2 was identified in a screen for Atg32 phosphorylation. Its role was confirmed by in vitro kinase assays and in vivo interaction with Atg32. CK deficient cells have a defect in mitophagy. The experiments are properly controlled and contribute to a general understanding in the regulation of selective autophagy. This is a solid study.

Comments to authors

1) Upon starvation Atg32 interaction with Atg11 is stimulated. The authors suggest that phosphorylating of Atg32 by CK2 induces the interacting with Atg11 and I suppose also with Atg8.

The authors can test this hypothesis with Co-IP experiments and this would improve the quality and impact of the study.

Referee #2:

Mitophagy mediates degradation of mitochondria via the autophagy pathway, and therefore is crucial for quality control of this organelle, and its aberration can lead to neurodegeneration as seen in Parkinson's disease. Receptor proteins that allow the autophagy machinery to recognize mitochondria to be eliminated have been found in yeast and mammals. Kanki's group identified Atg32 as a mitophagy-specific receptor in Saccharomyces cerevisiae and has unveiled the fundamental mechanism by which Atg32 triggers mitophagy. They have also reported that Atg32 is phosphorylated under mitophagy-inducing conditions, which is important for mitophagy induction through increasing its interaction with the adaptor protein Atg11. However, a kinase responsible for Atg32 phosphorylation has been unknown. In this study, Kanki et al. clearly showed that Atg32 phosphorylation and mitophagy are defective in casein kinase 2 (CK2) mutants or wild-type cells treated with an inhibitor specific to CK2. The authors also showed that CK2 directly interacts with and phosphorylates Atg32. Thus, they identified CK2 as the kinase that phosphorylates Atg32 to initiate mitophagy. This finding provides significant insights into the regulation of mitophagy, which is of great interest to the broad readership of EMBO reports. I made several comments and suggestions below, including the requests of additional experiments that will further strengthen the conclusion of this manuscript, which should be addressed by the authors before acceptance of the manuscript.

Specific comments:
(1) The authors should describe basic information on Cka1/2 and Ckb1/2 some more (e.g., Are these completely redundant or implicated in different events? What are the known physiological roles of CK2? What complex(es) do Ckas and Ckbs form? How do Ckbs serve as regulators of Ckas? (Do they stimulate Cka kinase activity or determine substrate specificity?).

(2) I wondered if CK2 mutants used in Figure 1 show any abnormality in growth and mitochondria morphology.

(3) The authors should examine whether the Cvt pathway is defective or not in CK2 mutants and wild-type cells treated with TBB.

(4) In Figure 4A, is the addition of Ckb1 effective on Cka1-mediated phosphorylation of Atg32? Whereas, as the authors described, the results in Figure 1 did not clearly show whether Ckbs are involved in mitophagy or not, answering to this question may clarify this point.

(5) Are Ser114 and Ser119 of Atg32 within the CK2 phosphorylation motif?

(6) The authors have not shown direct evidence of the phosphorylation of Ser114 and Ser119 (such as mass spectrometry identification); another possibility would be that these residues are involved in interaction with CK2. It should be confirmed that the SA mutations do not affect Atg32 interaction with CK2.

(7) The authors previously reported that Ser114 and Ser119 of Atg32 are important for its interaction with Atg11. They should show in this study that Atg32-Atg11 interaction is abolished in CK2 mutants and TBB-treated wild-type cells.

(8) Based on the results shown in Figures 4D and S5, the authors concluded that CK2 interacts with Atg32 under mitophagy-inducing conditions. However, the experiments were performed using cells shifted from SMD to SD-N but not from YPL. Does this indicate that CK2 interacts with Atg32 under nitrogen starvation conditions rather than mitophagy-inducing conditions?

(9) It would be interesting to describe the localization of CK2 under mitophagy-suppressing and inducing conditions.

Referee #3:

The authors have previously published that Atg32 is phosphorylated at Serine 114 and 119 during nitrogen-starvation induced mitophagy in yeast and the phosphorylation is essential for Atg32-Atg11 interaction and mitophagy. In this manuscript, they identified CK2 as a responsible kinase for the Atg32 phosphorylation. As the CK2-depletion in yeast resulted in the mitophagy deficiency, CK2 phosphorylation to Atg32 during nitrogen-starvation induced mitophagy is essential for the process. Although their findings are intriguing and important to understand the molecular mechanism for mitophagy in yeast, several problems should be solved before the publication.

Major concerns,
As CK2 has a constitutive kinase activity, it is unclear why Atg32 is NOT constitutively phosphorylated. Although they showed that CK2 binds to Atg32 during mitophagy, why does CK2 bind to Atg32 when the mitophagy is induced? In order to prove their model that CK2 regulates the mitophagy though Atg32 phosphorylation, the authors should show whether CK2 kinase activity is indeed increased during nitrogen-starvation condition or Atg32 has a change to allow efficient CK2 binding.

In mammal, it is believed that mitophagy is induced when the mitochondria gets damaged. Although nitrogen-starvation induces Atg32-dependent mitophagy, does depolarization or oxidative stresses against mitochondria also induce CK2 dependent mitophagy?

In vitro kinase assay clearly showed that CK2 is able to phosphorylate Atg32 directly. However, although they used mutant Atg32 both S114 and S119 are substituted to alanine to confirm the phosphorylation sites, it is unclear which residues are actually phosphorylated. They have to use single mutant for in vitro phosphorylation assay, instead of double mutant, and identify the site(s).

If Atg32 phosphorylation by CK2 is essential for the entering mitophagy process, overexpression of CK2a subunit, which has constitutive activity, should enhance mitophagy without nitrogen-starvation. As mammalian CK2a can phosphorylate S114 and/or S119 of Atg32 in vitro, it is worth test this possibility to confirm their model.

Minor problems,

In page 5, they described that "we screened kinase, kinase cofactor and kinase-related gene knockout strains". They should show the list of all tested kinases and cofactors.

In figure 4, does the S114A mutant have weak affinity with CK2? The authors should discuss why CK2-Atg32 interaction is enhanced by nitrogen-starvation.

1st Revision - authors' response 13 June 2013

Please find enclosed our revised manuscript (EMBOR-2013-37156V1) entitled “Casein kinase 2 plays an important role in mitophagy induction” by Tomotake Kanki, Yusuke Kurihara, Xiulian Jin, Tadahiro Goda, Yusuke Ono, Masamune Aihara, Yuko Hirota, Tetsu Saigusa, Yoshimasa Aoki, Takeshi Uchiumi, and Dongchon Kang, which was submitted for publication in *EMBO reports.*

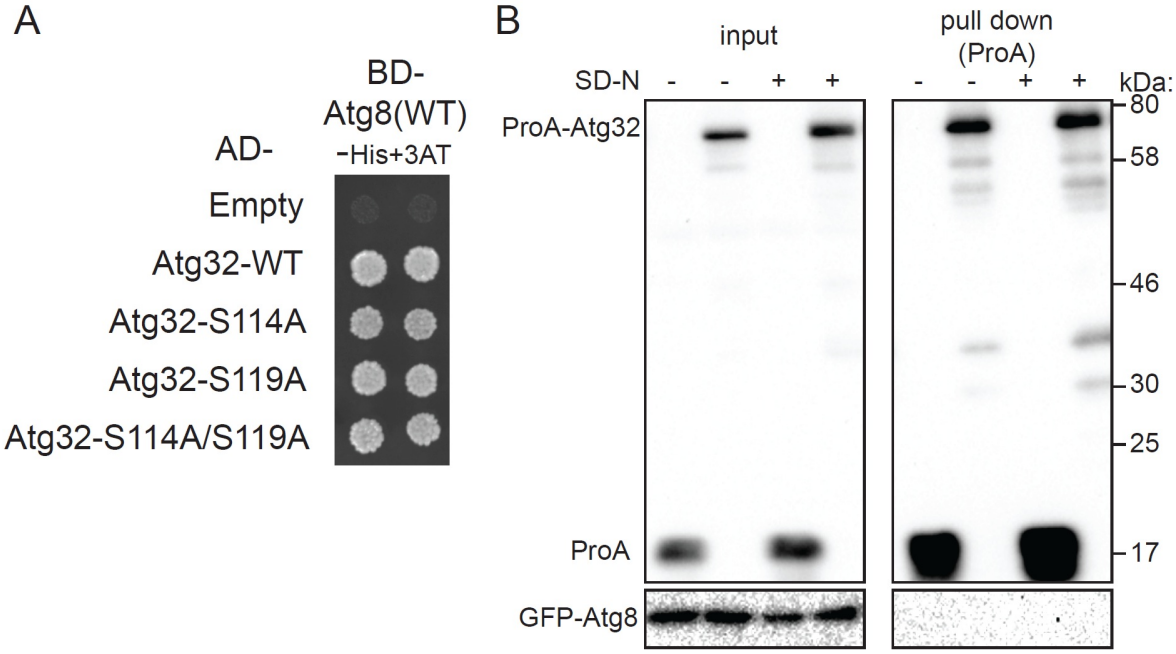
We thank the reviewers for their careful reading of our study and for their suggestions. The revised manuscript addresses each of the reviewers’ comments, as described below.

Reviewer #1

1) Upon starvation Atg32 interaction with Atg11 is stimulated. The authors suggest that phosphorylating of Atg32 by CK2 induces the interacting with Atg11 and I suppose also with Atg8. The authors can test this hypothesis with Co-IP experiments and this would improve the quality and impact of the study.

We thank the reviewer for this helpful comment. It is reasonable to propose that the Atg32–Atg11 interaction is CK2 dependent, and we showed that the interaction was substantially decreased in the CK2 temperature-sensitive mutant and in wild-type cells after TBB treatment (Supplementary Fig. S5A, B).

The Atg32–Atg8 interaction was observed by Okamoto's group by yeast-two-hybrid (Y2H) and immunoprecipitation (IP) (Okamoto K *et al.* (2009) *Dev Cell* 17: 87-97, Kondo-Okamoto N *et al. J Biol Chem* (2012) 287:10631-10638). As shown below (panel A), we were able to observe the Atg32–Atg8 interaction by Y2H. This interaction was not affected by the S114A or S119A mutation on Atg32. However, we could not observe the Atg32–Atg8 interaction by IP (panel B). To observe the Atg32–Atg8 interaction, Okamoto's group cultured cells in non-fermentable medium for 33 hours to grow cells to the stationary phase, which can induce mitophagy, and then performed IP. On the other hand, we used a short nitrogen starvation period (up to 6 hours) to induce mitophagy, because it was impossible to culture cells for a long time in CK2-suppressed conditions. This difference in culture conditions may cause the difference between Okamoto's results and our own. Another possibility is that the Atg32–Atg8 interaction is much weaker than the Atg32–Atg11 interaction. We pulled down ProA-Atg32 from whole-cell homogenates. By this method, we were able to observe co-precipitation of Atg11 but not of Atg8. Okamoto's group used a mitochondrial-enriched fraction for IP and this may make it possible to detect the weak Atg32–Atg8 interaction. Thus, we were not able to observe the requirement for CK2 activity for the Atg32–Atg8 interaction. Because the Atg32 S114A and S119A mutation did not affect the Atg32–Atg8 interaction by Y2H, it is highly possible that CK2 is not required.



2) The manuscript needs correcting for English grammar in some places.

We agree that our initial manuscript needed English language editing. The current revised manuscript has been edited by a native English speaker through an English language-editing service.

Reviewer #2

1) The authors should describe basic information on Cka1/2 and Ckb1/2 some more (e.g., Are these completely redundant or implicated in different events? What are the known physiological roles of CK2? What complex(es) do Ckas and Ckbs form? How do Ckbs serve as regulators of Ckas? (Do they stimulate Cka kinase activity or determine substrate specificity?).

We agree with the reviewer that it is important to describe the basic information on CK2. We have now included this information in the Introduction (from page 4, line 13 to page 5, line 6).

2) I wondered if CK2 mutants used in Figure 1 show any abnormality in growth and mitochondria morphology.

We observed the cell growth and mitochondrial morphology of the CK2 mutants (Supplementary Figs. S3 and S4). All the mutants showed slight growth suppression compared with WT cells in both YPD and YPL medium. In particular, the *ckb1Δ*, *ckb2Δ*, and *ckb1Δ/ckb2Δ* strains showed a growth defect in YPL medium. There were no mitochondrial morphological differences between the WT and any of the mutants.

3) The authors should examine whether the Cvt pathway is defective or not in CK2 mutants and wild-type cells treated with TBB.

We agree with the reviewer that the Cvt pathway, one of the selective autophagy pathways, should be tested in CK2-inhibited cells. We examined ApeI maturation in CK2-mutant or -WT cells treated with TBB. In both types of cells, ApeI maturation was normal, suggesting that CK2 activity is not required for the Cvt pathway (Supplementary Fig. S6B, C).

4) In Figure 4A, is the addition of Ckb1 effective on Cka1-mediated phosphorylation of Atg32? Whereas, as the authors described, the results in Figure 1 did not clearly show whether Ckbs are involved in mitophagy or not, answering to this question may clarify this point.

We agree with the reviewer that this experiment is important to determine the role of Ckb1 on Atg32 phosphorylation. We examined Atg32 phosphorylation by Cka1 supplemented with or without Ckb1 *in vitro*. Unexpectedly, supplementation with Ckb1 partially suppressed Atg32 phosphorylation *in vitro* (Supplementary Fig. S8C). This finding might suggest that Ckb1 negatively regulates Atg32 phosphorylation. On the other hand, Atg32 phosphorylation was partially suppressed in the *ckb1*Δ strain, suggesting that Cbk1 positively regulates Atg32 phosphorylation (Fig. 1B). To date, we do not have a conclusive answer to explain this discrepancy. One possible explanation is that because Ckb1 is a substrate of Cka1, Ckb1 competitively blocks Atg32 phosphorylation *in vitro*. Further studies are needed.

5) Are Ser114 and Ser119 of Atg32 within the CK2 phosphorylation motif?

The minimum consensus motif for phosphorylation by CK2 is SXXE/D, although there are many exceptions (Meggio F and Pinna LA (2003) *FASEB J.* 17:349-68). Both Ser114 and Ser119 on Atg32 have this motif (114S-S-S-D and 119S-E-E-E). We have now described this motif on page 12 line 12.

6) The authors have not shown direct evidence of the phosphorylation of Ser114 and Ser119 (such as mass spectrometry identification); another possibility would be that these residues are involved in interaction with CK2. It should be confirmed that the SA mutations do not affect Atg32 interaction with CK2.

We agree with the reviewer that it is important to determine whether the Atg32-2SA mutation affects the Atg32–CK2 interaction. We compared the Cka1 interaction with Atg32-WT and Atg32-2SA by protein A affinity pull-down assay. As shown in Supplementary Fig. S9B, both Atg32WT and Atg32SA co-precipitated Cka1 to a similar level, suggesting that the Atg32-2SA mutation does not affect the Atg32–CK2 interaction.

7) The authors previously reported that Ser114 and Ser119 of Atg32 are important for its interaction with Atg11. They should show in this study that Atg32-Atg11 interaction is abolished in CK2 mutants and TBB-treated wild-type cells.

This comment is similar to comment 1) of reviewer #1. As shown for this former comment, we observed the Atg32–Atg11 interaction in CK2 mutants and TBB-treated wild-type cells (Supplementary Fig. S5A, B). As the reviewer expected, the Atg32–Atg11 interaction was suppressed by CK2 inactivation.

8) Based on the results shown in Figures 4D and S5, the authors concluded that CK2 interacts with Atg32 under mitophagy-inducing conditions. However, the experiments were performed using cells shifted from SMD to SD-N but not from YPL. Does this indicate that CK2 interacts with Atg32 under nitrogen starvation conditions rather than mitophagy-inducing conditions?

We agree with the reviewer that our initial analysis of the Atg32–CK2 interaction did not completely mimic mitophagy-inducing conditions. Thus, we pre-cultured cells in non-fermentable SML medium, then shifted to SD-N, and observed the Atg32–CK2 interaction by pull-down assay. Unexpectedly, we could not observe the Atg32–CK2 interaction under these conditions (Supplementary Fig. S9B). Although we went to great lengths to observe the interaction, such as using different strains or other pull-down methods, we could not observe the Atg32–CK2 interaction reproducibly if cells were pre-cultured in SML and then shifted to SD-N. In contrast, we could reproducibly observe the Atg32–CK2 interaction when cells were pre-cultured in SMD medium (as shown in Fig. 4D and Supplementary Fig. S9A). It is generally thought that enzyme–substrate interactions are weak and occur only transiently; thus, they are sometimes difficult to observe. In the case of the Atg32–CK2 interaction, only when cells are pre-cultured in SMD medium might the Atg32–CK2 interaction become sufficiently stable or efficient to be observed. Because we could not obtain conclusive evidence of the Atg32–CK2 interaction, we decided to tone down our conclusion that CK2 has the potential to interact with Atg32 (from page 13, line 1 to page 14, line 2).

9) It would be interesting to describe the localization of CK2 under mitophagy-suppressing and inducing conditions.

We agree with the reviewer that it is important to observe the localization of CK2, and especially whether CK2 colocalizes with mitochondria when mitophagy is induced. CK2 is thought to be a constitutively active kinase and it is unclear how Atg32 phosphorylation by CK2 is regulated. One possibility is that CK2 accumulates in mitochondria to phosphorylate Atg32 under mitophagy-inducing conditions. To observe the localization of CK2, we tagged GFP onto each of the CK2 components (Cka1-GFP, Cka2-GFP, Ckb1-GFP, and Ckb2-GFP) and observed their localization before and after mitophagy induction. When cells were cultured in YPD or YPL, the majority of CK2 localized in the nucleus, while some CK2 diffused within the cytoplasm (Supplementary Figs. S10, S11A, and S12A). After mitophagy induction by starvation, the majority of CK2 remained localized in the nucleus and did not accumulate in mitochondria (Supplementary Figs. S11B and S12B). Because CK2 is an abundant protein and a proportion of CK2 is always present in the cytoplasm, it might be difficult to detect mitochondrially localized CK2 that transiently interacts with and phosphorylates mitochondrial Atg32 (from page 15, line 18 to page 16, line 13).

Reviewer #3

1) As CK2 has a constitutive kinase activity, it is unclear why Atg32 is NOT constitutively phosphorylated. Although they showed that CK2 binds to Atg32 during mitophagy, why does CK2 bind to Atg32 when the mitophagy is induced? In order to prove their model that CK2 regulates the mitophagy though Atg32 phosphorylation, the authors should show whether CK2 kinase activity is indeed increased during nitrogen-starvation condition or Atg32 has a change to allow efficient CK2 binding.

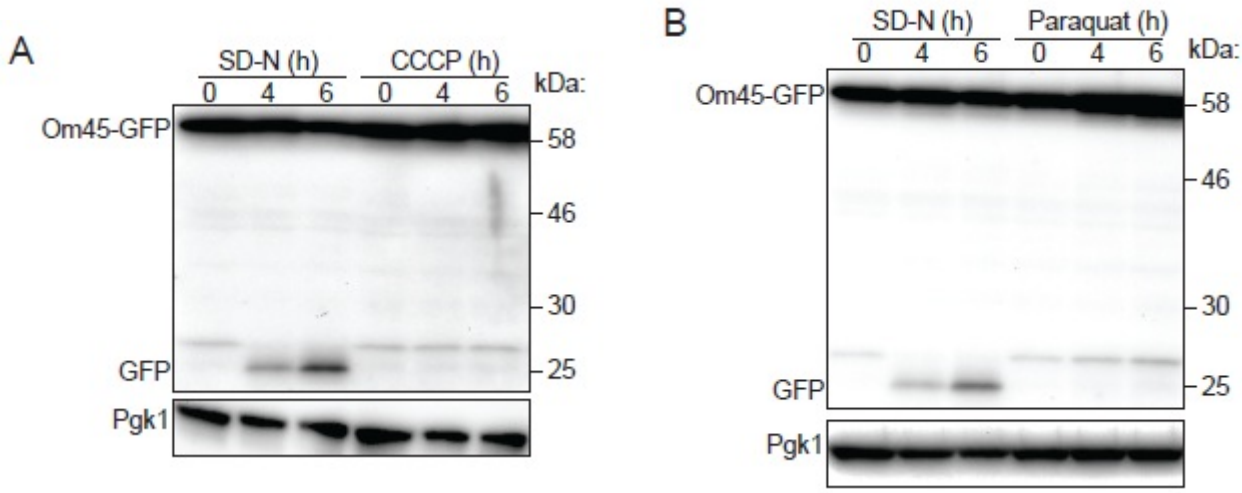
We agree that, in our original manuscript, it was unclear how Atg32 phosphorylation by CK2 is regulated. Accordingly, we carried out two experiments. We first tried to identify the region in Atg32 that interacts with CK2. We expressed several lengths of Atg32 deletion mutants and observed the Atg32–CK2 interaction by pull-down assay. However, because most Atg32 deletion mutants did not interact with CK2, we could not identify the specific CK2 interaction region. Next, we speculated that CK2 accumulates in mitochondria and phosphorylates Atg32 to initiate mitophagy, and decided to observe the localization of CK2. As noted in our response to comment 9) of reviewer #2, we were able to observe GFP-tagged CK2 components before and after mitophagy induction, but we could not obtain any evidence that CK2 accumulates in mitochondria before and after mitophagy induction (Supplementary Figs. S10, S11, S12).

CK2 is a well-studied kinase and is known to phosphorylate more than 300 substrates. However, it is still not clear how the phosphorylation of individual substrates is regulated. We think

that a complete analysis of the CK2 regulatory mechanisms is beyond the scope of our present analysis.

2) In mammal, it is believed that mitophagy is induced when the mitochondria gets damaged. Although nitrogen-starvation induces Atg32-dependent mitophagy, does depolarization or oxidative stresses against mitochondria also induce CK2 dependent mitophagy?

We agree with the reviewer that it would be of additional interest to demonstrate that damaged mitochondria are degraded by mitophagy. To answer this question, we depolarized mitochondria by treating cells with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), or added oxidative stress by treating cells with paraquat, and observed mitophagy. As shown below, treatment with neither CCCP (20 μM) nor paraquat (1 mM) induced mitophagy in *S. cerevisiae*. Based on our previous observations, we believe that mitophagy is most efficiently observed not by mitochondrial damage but when cells are shifted from non-fermenting growth to fermenting growth in yeast (Kurihara Y *et al.* (2012) *J Biol Chem* **287:** 3265-3272). However, our findings do not exclude the possibility that damaged mitochondria are eliminated by mitophagy in yeast. Further studies are needed.



3) In vitro kinase assay clearly showed that CK2 is able to phosphorylate Atg32 directly. However, although they used mutant Atg32 both S114 and S119 are substituted to alanine to confirm the phosphorylation sites, it is unclear which residues are actually phosphorylated. They have to use single mutant for in vitro phosphorylation assay, instead of double mutant, and identify the site(s).

We agree with the reviewer that this experiment is important to determine the efficiency of phosphorylation of Ser114 and Ser119. We made recombinant GST-tagged Atg32WT, Atg32-S114A, Atg32-S119A, and Atg32-S114AS119A(2SA) and observed whether they are phosphorylated by recombinant yeast CK2 or human CK2 *in vitro*. As shown in Fig. 4B and C, both Atg32-S114A and Atg32-S119A were phosphorylated *in vitro*, but Atg32-2SA was not. In addition, S119 was more efficiently phosphorylated than S114 *in vitro*. This is consistent with our previous finding that Ser119 was dominantly phosphorylated while Ser114 was subordinately phosphorylated *in vivo* (Aoki Y *et al.* (2011) *Mol Biol Cell* **22:** 3206-3217).

4) If Atg32 phosphorylation by CK2 is essential for the entering mitophagy process, overexpression of CK2a subunit, which has constitutive activity, should enhance mitophagy without nitrogen-starvation. As mammalian CK2a can phosphorylate S114 and/or S119 of Atg32 in vitro, it is worth test this possibility to confirm their model.

We agree with the reviewer that it would be interesting to test whether overexpression of CK2 enhances mitophagy. We overexpressed Cka1 or Cka1/Ckb1 in WT cells and observed mitophagy. Unexpectedly, and as shown in Supplementary Fig. S5C, we found that overexpression of CK2 does not affect mitophagy. There may be several factors that regulate the amount of mitophagy, other than CK2-dependent Atg32 phosphorylation.

5) In page 5, they described that "we screened kinase, kinase cofactor and kinase-related gene knockout strains". They should show the list of all tested kinases and cofactors.

We have produced a list of genes that we tested (Supplementary Table S1).

6) In figure 4, does the S114A mutant have weak affinity with CK2? The authors should discuss why CK2-Atg32 interaction is enhanced by nitrogen-starvation.

This comment is similar to comment 6) of reviewer #2. As shown for this former comment, the Atg32-2SA mutant showed a similar affinity for CK2 (Supplementary Fig. S9B). Although we do not have a conclusive answer as to why the Atg32–CK2 interaction is enhanced by nitrogen starvation, we have now discussed several possibilities (from page 15, line 18 to page 16, line 13).

We hope that we have adequately addressed all the comments that the reviewers raised. We thank you and the reviewers for your useful, considerable, and important comments. We hope that the revised manuscript is now suitable for publication.

2nd Editorial Decision 28 June 2013

Thank you for your patience while we have reviewed your revised manuscript. Referee 2 was unfortunately unavailable to assess the revised version of your study. However, as you will see from the reports below, both referees 1 and 3 are now supportive of its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

In going through your manuscript prior to acceptance, I have noted that, at over 35,000 characters, the text is longer than we can accommodate. You will thus need to shorten it to a maximum of 30,000 characters, including spaces and figure legends. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which will help eliminate the redundancy that is inevitable when discussing the same experiments twice. Please note, however, that the materials & methods section cannot be shortened any further.

In addition, the information regarding the number of independent experiments performed and the identity of the error bars shown in the graphs needs to be included in the legends to figures 3A, 3B and Supplementary figure 6.

In supplementary figure 4B, right, the photos that should depict delta-cka2 and delta-ckb2 seem identical. Please check and provide the relevant data.

Also, the contrast of the gels shown in figures 1A, 1B, 4D right, and Supp figure 7 is too high. Please provide versions that have more reasonable contrast settings, as those provided in the rest of the figures.

We encourage the publication of original source data -particularly for electrophoretic gels and blots- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF 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.

Lastly, as a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature and let me know if you do NOT agree with any of the changes.

After all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

****
Edited title and abstract

Casein kinase 2 is essential for mitophagy

Mitophagy is a process that selectively degrades mitochondria. When mitophagy is induced in yeast, the mitochondrial outer membrane protein Atg32 is phosphorylated, interacts with the adaptor protein Atg11, and is recruited into the vacuole with mitochondria. We screened kinase-deleted yeast strains and found that CK2 is essential for Atg32 phosphorylation, Atg32-Atg11 interaction and mitophagy. Inhibition of CK2 specifically blocks mitophagy, but not macroautophagy, pexophagy or the Cvt pathway. In vitro, CK2 phosphorylates Atg32 at serine 114 and serine 119. We conclude that CK2 regulates mitophagy by directly phosphorylating Atg32.

****

REFEREE REPORTS:

Referee #1:

I have read the response by the authors to my previous comments and I am satisfied that they have dealt with them appropriately.
I have no further concerns.

Referee #3:

For revised manuscript, the authors responded to the reviewers' concerns as possible as they can. The reviewer thinks it is possible to publish now.

2nd Revision - authors' response 07 July 2013

Please find enclosed our shortened manuscript entitled “Casein kinase 2 is essential for mitophagy” by Tomotake Kanki, Yusuke Kurihara, Xiulian Jin, Tadahiro Goda, Yusuke Ono, Masamune Aihara, Yuko Hirota, Tetsu Saigusa, Yoshimasa Aoki, Takeshi Uchiumi, and Dongchon Kang, which was submitted for publication in *EMBO reports*.

We combined Results and Discussion, shortened the manuscript, and changed the title and abstract following your suggestions. During this process, we changed the order of supplementary figures (previous figures S7, S8, and S9 are now figure S9, S7, and S8, respectively). We added the information regarding the number of independent experiments and the error bars in the legends to figures 3A, 3B and supplementary figure S6A, corrected supplementary figure 4B, and changed the contrast of the gels shown in figures 1A, 1B, 4D and supplementary figure S9.

We hope that we have adequately addressed your comments and that this revised manuscript is now suitable for publication.

3rd Editorial Decision 11 July 2013

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.

