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Hrr25 kinase promotes selective autophagy by phosphorylating the cargo-receptor Atg19

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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.)

Editor: Nonia Pariente

1st Editorial Decision 06 May 2014

Thanks for your patience while your study was peer-reviewed. We have now received the three enclosed reports on it and I am happy to say that, as you will see, all referees are highly supportive of publication in EMBO reports. Referees 1 and 3 nevertheless do raise some issues that need to be addressed in a round of minor revision before the study can be accepted for publication.

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. The revised study will be sent back to referees 1 and 3 for a quick check before a final decision is made.

Do get in touch with me if I can be of any help during the revision process. I look forward to receiving the final version of your study as soon as possible.

REFEREE REPORTS:

Referee #1:

Kraft and coworkers report here in on a new and interesting link between the Hrr25 kinase, know to regulate multiple cellular processes, and selective autophagy. Both Atg19 and Atg34 were phosphorylated by this kinase. By utilizing mas spectrometry approach of samples obtained from different yeast strains the authors identified multiple phosphorylation sites at the Atg11 binding region of Atg19 and Atg34. Phosphorylation of Atg19 in this region was essential for the Ape1 maturation while phosphorylation of Atg34 was found important for Ams1 trafficking into the vacuole. The authors also provide evidence that Atg19 phosphorylation promoted its interaction with the Atg11 promoting its interaction with its cargo.
Study of the receptors that mediate the selective recruitment of cargo for autophagic degradation is both important and interesting. The authors' hypothesis is well based and is mostly supported by the presented data.

The authors should test the expression of the atg11D211A in background of ATG1 knockout strain or provide an explanation why not doing this.

In the permissive temperature of the hrr25-ts strain there are relatively few cells with ATG11 dots. This should be addressed.

AMS1 delivery into the vacuole should be tested also in the hrr25-ts strain.

In figure 4G the background of the control autoradiograph looks different from the rest of the experiment. The authors should clarify this issue.

Referee #2:

In this manuscript the authors show that the CKI kinase family member Hrr25, phosphorylates Atg19, a cargo receptor on the Cvt pathway that interacts with Atg11. The phosphorylation of Atg19 by Hrr25 is required for its interaction with Atg11. The authors also show that Atg34, another cargo receptor, is a substrate for Hrr25. Understanding how cargo receptors are phosphorylated to promote their interaction with the autophagy machinery is an important question in the autophagy field. The experiments in this manuscript are nicely done and prove the point the authors claim.

Referee #3:

The authors of this short reports show that the Hrr25 kinase directly phosphorylates Atg19, the cargo receptor of the Cvt pathway. This modification is essential for the subsequent recruitment of the Atg11 adaptor protein, a step essential for the progression of the Cvt pathway.
The authors also show that another cargo receptor, Atg34, is also modified and regulated similarly.

Major:

Figure 2A , 2C and 4F. These experiments have to be repeated without the overexpression of Ape1. This latter system is an artificial system and while the (speculative) hypothesis is that the Cvt vesicle formation is blocked because the isolation membrane is not able enwrapped it. Another possible interpretation, which is not mutually exclusive, is that overexpression of Ape1 could affect the downstream interactions of Atg19 and/or Atg11 with factor such as Atg1.
Endogenous Ape1 can be easily imaged and in repeating the experiment, the authors have to also

stain the vacuole (blue dyes for this organelle exist). Atg11 is required for Ape1 oligomer recruitment next to the vacuolar surface to trigger the PAS formation and quantification of Ape1 oligomer localization in regard to the vacuole will provide additional supporting evidence that Atg11 cannot be engaged in the Cvt pathway when Atg19 is not phosphorylated.

Figure 3. I could not find in the Results and Methods sections whether the phosphorylation sites of Atg34 were determined using a HTB-Atg34 fusion protein isolated from cells grown in rich medium or starved. In the context of this paper and to understand the role of Hss25, it would be very informative to analyze the phosphorylation status of the analyzed Ser residues of the Atg19 and Atg34 in presence or absence of nutrients.

Minor:

First paragraph of the introduction. Some references must be added.

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Figure 1. Why the role of Atg19 Thr195, which is also phosphorylated, has not been analyzed?

Correspondence - authors 08 May 2014

It is absolutely no problem to do these experiments and even gives us the time to do the additional things we would like as controls added. We went through all the setup so if we repeat Fig 2A and 4F as requested without Ape1 ox, we should manage maybe even by end of June. The reviewer also requested the replacement for 2C. This would take us quite long as we don't have the strains ready. But if we do it for 2A, that should already clarify his point with the proximity of Ape1 to the vacuole, and as 2A shows also Atg11, and Atg8 is well known to be downstream of Atg11, we don't really need that, or? If you think we should still do 2C also, it probably will take us at least until end of July, but would be easily doable. Please let me know if you think it is fine without 2C. All other requests we can do.

Editor response:

I agree with you that if you can repeat figures 2A and 4F without Ape1 over-expression, this would suffice. Generating the strains just for 2C seems not to be worth the resources and time, considering the value it would add to the paper.

1st Revision - authors' response 30 May 2014

Response to the points raised by the referees.

We thank all referees for their insightful comments, which helped us to improve our manuscript. As explained in detail below, we have addressed all points and added extensive new data including additional validation experiments:

• In addition to the experiments described in the point-to-point reply below, we also analyzed phosphorylation on Atg19 in hrr25-ts cells by quantitative mass spectrometry (SILAC). As expected, phosphorylation in the Atg11 binding region on S390, 391 and 369 was detected when Atg19 was isolated from wild type cells, but was decreased on these sites in hrr25-ts mutants, supporting that Hrr25

phosphorylates these residues on Atg19 in vivo. These results are now shown in Figure E4A.
• We also show that the Cvt defect in the hrr25-ts strain is indeed due to the loss of Hrr25 function by rescuing Cvt activity in an hrr25-ts strain with a centromeric plasmid containing wild type HRR25. These results are now shown in Figure 4A.
• Furthermore we show that mimicking phosphorylation on Atg19 in the Atg11 binding region (Atg19-3D) can partially bypass the requirement for Hrr25 (new Figure 4B).

Referee #1:

Kraft and coworkers report here in on a new and interesting link between the Hrr25 kinase, know to regulate multiple cellular processes, and selective autophagy. Both Atg19 and Atg34 were phosphorylated by this kinase. By utilizing mas spectrometry approach of samples obtained from different yeast strains the authors identified multiple phosphorylation sites at the Atg11 binding region of Atg19 and Atg34. Phosphorylation of Atg19 in this region was essential for the Ape1 maturation while phosphorylation of Atg34 was found important for Ams1 trafficking into the vacuole. The authors also provide evidence that Atg19 phosphorylation promoted its interaction with the Atg11 promoting its interaction with its cargo.
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The authors should test the expression of the atg11D211A in background of ATG1 knockout strain or provide an explanation why not doing this.

We agree with the referee that it is important to show expression levels of ATG1 wild type and mutant. In the atg1-D211A strain used, the kinase-dead allele replaces the wild type gene genomically at its native locus. We previously confirmed its proper expression levels (Papinski et al., Mol Cell 2014) and now included panels showing the expression levels comparing Atg1 wild type and the D211A mutant in figure E2C.

In the permissive temperature of the hrr25-ts strain there are relatively few cells with ATG11 dots. This should be addressed.

We thank the reviewer for this comment. Indeed, the hrr25-ts strain is defective for Cvt also at the permissive temperature: compared to wild type, Atg11 dot formation is reduced by 75% and Ape1 maturation is strongly impaired. This defect becomes even more pronounced at the restrictive temperature, at which virtually no Atg11 dots are formed and Ape1 maturation is further reduced. We now clarified this in the text:

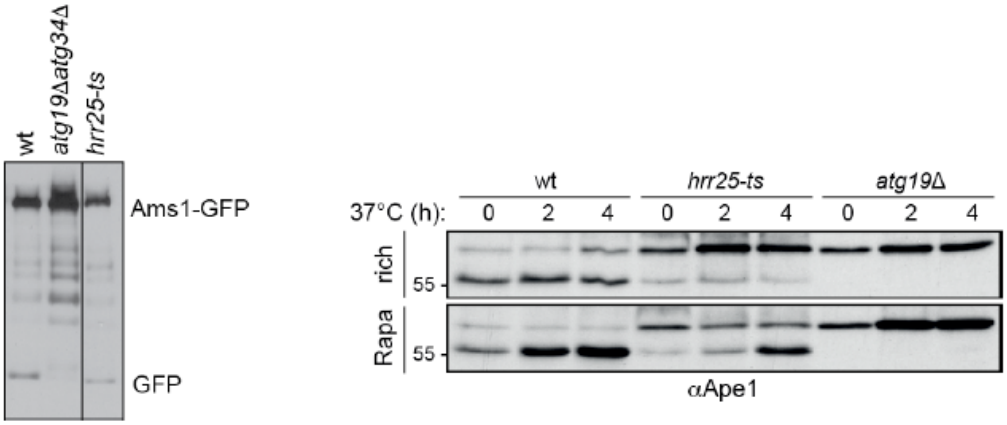
"hrr25-ts mutant cells already showed a severe Cvt pathway defect at 18ºC, indicating that Hrr25 kinase function is strongly impaired also at the permissive temperature."

In addition, we now added a panel in Figure 4A showing that this defect is caused specifically by the loss of Hrr25 function, by rescuing the mutant phenotype with a centromeric plasmid containing Hrr25.

AMS1 delivery into the vacuole should be tested also in the hrr25-ts strain.

As suggested by the reviewer we analyzed Ams1-GFP cleavage in the hrr25-ts strain and compared it to the wild type. Ams1-GFP is still processed in an hrr25-ts strain, possibly less than in the wild-type (see figure below). We also observed that Ape1 processing in hrr25-ts cells is rescued to some degree upon rapamycin treatment. These findings could indicate that Atg19 and Atg34 phosphorylation can be at least partially performed by another kinase upon starvation, whereas Hrr25 is essential for the phosphorylation under rich conditions. However, also bulk autophagy might contribute to the processing under starvation conditions. Extensive further experiments such as quantitative mass spectrometric analyses are required to fully understand this regulatory mechanism and the role of Hrr25 upon starvation. Therefore, these preliminary findings were not incorporated

into this manuscript, but will be followed up in future studies.



In figure 4G the background of the control autoradiograph looks different from the rest of the experiment. The authors should clarify this issue.

Based on the reviewer's comment, we realized that the experiment was not explained clearly in the text. The autoradiographs shown for Atg19 using Hrr25 or a control come from the same gel with the same exposure, which we now state in the figure legend.

"Both Atg19 panels are from the same gel with the same exposure."

As a kinase dead version of Hrr25 is not viable and the co-expression of a tagged version over the wild type gene is also not an option due to self-interaction of Hrr25 (Petronczki et al, Cell 2006), we used as a negative control an immunoprecipitation from a non-tagged strain as well as for a substrate control recombinant GST and the nonphosphorylatable Atg19-3A. The background in the Hrr25 lanes most likely results from Hrr25 activity on co-precipitating proteins, whereas no kinase is present in the control immunoprecipitation. We have now clarified this in the Expanded View Methods section as follows:

"As a kinase dead version of Hrr25 is not viable and could therefore not be used as a negative control (Petronczki et al, Cell 2006), we used instead an immunoprecipitation of a non-tagged strain. The co-expression of a tagged Hrr25-kinase dead version with the endogenous wild type gene is also not an option as Hrr25 self-interacts, which would result in the co-precipitation of the active wild-type kinase in the kinase-dead samples."

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Figure 2A , 2C and 4F. These experiments have to be repeated without the overexpression of Ape1. This latter system is an artificial system and while the (speculative) hypothesis is that the Cvt vesicle formation is blocked because the isolation membrane is not able enwrapped it. Another possible interpretation, which is not mutually exclusive, is that overexpression of Ape1 could affect the downstream interactions of Atg19 and/or Atg11 with factor such as Atg1.
Endogenous Ape1 can be easily imaged and in repeating the experiment, the authors have to also stain the vacuole (blue dyes for this organelle exist). Atg11 is required for Ape1 oligomer recruitment next to the vacuolar surface to trigger the PAS formation and quantification of Ape1 oligomer localization in regard to the vacuole will provide additional supporting evidence that Atg11 cannot be engaged in the Cvt pathway when Atg19 is not phosphorylated.

We thank the referee for suggesting how to further strengthen our findings. Whereas we are aware of the fact that the overexpression of Ape1 is an artificial system, we believe it is a valuable tool to monitor interactions in vivo. As requested by the reviewer, we now included new experiments using endogenous Ape1 and vacuolar staining. The new results presented in Figures E2A and E4B support our previous findings and clarify that Ape1 is localized as expected in Atg19 mutant and hrr25-ts cells.

Figure 3. I could not find in the Results and Methods sections whether the phosphorylation sites of Atg34 were determined using a HTB-Atg34 fusion protein isolated from cells grown in rich medium or starved. In the context of this paper and to understand the role of Hss25, it would be very informative to analyze the phosphorylation status of the analyzed Ser residues of the Atg19 and Atg34 in presence or absence of nutrients.

We thank the referee for noting this and apologize for the imprecise description. Atg34 and Atg19 were analyzed both from rich and rapamycin treated cultures using SILAC. No difference could be detected in phosphorylation in the Atg11 binding region for Atg19. For Atg34 we did note a 6x upregulation of a doubly phosphorylated peptide: 373 ADALSSPDESSIMSTPFK 390. One phosphorylation could be clearly assigned to S382 and the other phosphorylation was ambiguously on either S377 or S378. A peptide carrying a single phosphorylation on either S377 or S378 was increased 2x upon rapamycin treatment. This suggests that S382 phosphorylation could account for a 3x increase. In non-quantitative mass spectrometric analyses we found S382 and S383 doubly phosphorylated peptides, confirming their existence in vivo. However, we could not detect this specific peptide in SILAC approaches. It should be noted here that mass spectrometric phosphorylation analyses of this Atg34 region is very intricate due to the presence of 6 phosphorylatable amino acids. Although all evidence points to an upregulation in phosphorylation on at least S382, we did not incorporate this in the manuscript for clarity. Figure 3 shows the phosphomap of rapamycin treated cells, which is stated in the figure legend. We also added this statement to the methods of the Expanded View section clarifying the situation:

"Both Atg34 and Atg19 were analyzed both from rich and rapamycin treated cultures using SILAC. No difference could be detected in phosphorylation in the Atg11 binding region for Atg19.

For Atg34 we did note a 6x upregulation of a doubly phosphorylated peptide: ADALSSPDESSIMSTPFK. One phosphorylation could be clearly assigned to S382 and the other phosphorylation was ambiguously on either S377 or S378. A peptide carrying a single phosphorylation on either S377 or S378 was increased only 2x upon rapamycin treatment. This suggests that S382 phosphorylation could account for a 3x increase. In non-quantitative mass spectrometric analyses we found S382 and S383 doubly phosphorylated peptides confirming their existence in vivo"

Minor:

First paragraph of the introduction. Some references must be added.

We now refer to a review in the first paragraph of the introduction.

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P3, L20. I would change "Genetic analyses..." into "Genetic screens..."
P4, L1. I would change "...by interactions with..." into "by subsequent interactions with..."

All three text changes have been incorporated as suggested by the reviewer.

Figure 1. Why the role of Atg19 Thr195, which is also phosphorylated, has not been analyzed?

As noted by the reviewer, we detected several other phosphorylation sites including T195 on Atg19. In this study we focused on the role of phosphorylation in the Atg11 binding region. The analysis of other phosphorylation sites on Atg19 is subject of future studies.

2nd Editorial Decision 10 June 2014

I have now had time to go through your revised version in detail and heard back from referees 1 and 3, who support publication and have no further comments. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

