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Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast

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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: Anne Nielsen

1st Editorial Decision 01 July 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the slight delay in communicating our decision to you. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express high interest in your findings; however, they do also raise a number of points and requests for additional data that will have to be addressed before they can support publication of a revised manuscript. While we do find all points raised by the authors to be valid and important, I would ask you to particularly focus your efforts on the following points:

-> Please provide further experimental data to address the relative contribution of selective vs general autophagy in the RNA degradation observed here (ref#1). This would also allow you to more extensively discuss and address the concerns raised by ref#1 about partial contradictions with earlier work on ribophagy.

-> Please conduct transcriptome analysis to address the extent and variety of cellular RNAs that would be subject to degradation via general autophagy (ref#2 point 1 and #3 point #1). This point is also reflected in the suggestion from ref#1 to further discuss the physiological implications of the reported findings.

-> In addition, I would strongly recommend that you follow the suggestion from ref#2 (point 2) and look at the generality of this pathway in the context of other autophagy-triggering stress conditions.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

The study by Huang et al. deals with starvation-induced RNA catabolism by autophagy. Utilizing metabolome, genetic and biochemical analysis, the authors identify the nucleotidase Pho8 and the ribonuclease Rny1 as the responsible enzymes for vacuolar RNA degradation. Additional data of this manuscript further allowed the identification of the nucleosidases Pnp1 and Urh1, responsible for the following break down into purine and pyrimidine in the cytosol. Furthermore, the manuscript provides evidence that the autophagic core machinery is essential while proteins involved in selective autophagy play a less significant role in this process. Interestingly, also Ubp3 and Bre5, proteins essential for ribophagy, the selective autophagic degradation of ribosomes, were found none essential for this process.
Taken together the data of this manuscript provide convincing evidence for starvation induced autophagic RNA degradation. The authors should address the concerns listed below and provide a more detailed discussion regarding the conflict with the reports on ribophay.

Concerns:

A more detailed characterization of the autophagic factors needed for this process should be presented to improve the authors' model.

Fig.1B: atg11 cells show a significant decrease in nucleoside levels, suggesting some aspects of selectivity. The authors should therefore discuss this in more details. Furthermore, this figure should also include the relative intensity of uridine after 3 hours of starvation to show the delayed drop indicated in figure 1 A.

Fig.2A: compared to figure 1 the relative intensity of adenosine starts already very high at 0 hours of starvation showing high standard deviation. These data lower the reliability of the assay.
Fig2B: The different levels of 5'NMPs and the resulting meaning should be better described in the text.

Fig 3C: testing the effect of atg2 rny1 double knockout would help solidify the conclusion of an autophagic dependent metabolism.

Fig.4: the authors should include morphological analysis of bre5 and ubp3 cells using the Gel Green dye to monitor free RNA in the vacuoles after starvation.

Fig5: The deviations in the mean values are missing.

Fig E2: A negative control e.g. atg2 is missing.

FigE5: As FigE5 contains mainly the data of figure 5 it should be included in figure 5 and statistical information should be added.

The authors mention Amd1 as a potential deaminase for generation of 3'IMP. This hypothesis should be checked in amd1 cells

A more elaborate discussion on the physiological meaning of vacuolar RNA catabolism should be given?

Minor

Fig E1: Is there any particular reason why this figure is lacking adenosine?

Figure E4 and E5 should be exchanged.

Line 399 of discussion should point to the figure 3 to make it easier to follow.

Referee #2:

In their manuscript 'Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast', Hanghang et al. present detailed matabolomic studies of autophagy-induced RNA degradation in S. cerevisiae and assay the transformations of the resulting metabolites. In addition, presented data are strengthened by analogous results obtained in S. pombe. The manuscript is well organized, consecutive experiments are introduced in an orderly and logical fashion and the figures are clear and well described. The observation that during nitrogen starvation RNA becomes degraded to nucleosides and bases via 3'-NMPs and the resulting products are further deaminated and excreted from the cells is of major basic biological interest - it is actually surprising that a topic of such importance has never before been this extensively tackled in the autophagy research. The following points need to be further addressed:

1) the authors suggest that non-selective autophagy (as opposed to ribophagy) is responsible for the vacuolar RNA degradation - in this context it would be interesting to see a comparison of degradation kinetics of some ribosomal proteins, chosen rRNA molecules (possibly from both subunits) and other intracellular RNA species (tRNA and mRNA) - this experiment could provide additional evidence that the nitrogen starvation-induced RNA degradation proceeds via non-selective bulk degradation of cytoplasmic constituents

2) as an addendum to the presented data obtained under nitrogen-starvation conditions, additional autophagic stimuli (different types of starvation or pharmacologic treatment) could be briefly tested (here analysis of nucleoside accumulation kinetics would suffice) - this would give a broader picture of autophagic RNA degradation and would be of great general interest

other remarks:
- in lines 133 and 164 the word 'significantly' is used in relation to results on which no statistical significance tests were performed - please add the missing statistics
- for data represented in Fig. E3B, p values should be included

- there's a marked discrepancy between adenosine levels depicted in Fig. 1B and 2A - I believe that it can be at least partially explained by the generally low levels of this metabolite in the cells (as shown in Fig. 5B) but still some word of clarification would be beneficial in order to avoid confusing the readers

Referee #3:

This manuscript describes the fate of cellular RNA during nitrogen starvation in Saccharomyces cerevisiae. The authors use LC/MS to follow intra- and extracellular nucleotide/nucleoside/base levels upon nitrogen starvation. The major findings presented in the manuscript are that, under nitrogen starvation conditions, nucleosides transiently accumulate in a manner dependent upon the core autophagy machinery. This accumulation is not observed in strains mutant for the phosphatase Pho8 or the ribonuclease Rny1, while nucleoside 3' monophosphates accumulate in the pho8 mutant. Pho8 and Rny1 are induced upon nitrogen starvation and accumulate in the vacuole, as does RNA. Nucleosides and in particular xanthine and uracil bases accumulate extracellularly upon nitrogen starvation. Finally, this pathway was shown to be conserved through to fission yeast. Based on their data the authors suggest a model wherein, upon nitrogen starvation, RNA is targeted to the vacuole where it is degraded by Rny1 and Pho8, further metabolized to its bases in the cytosol and ultimately excreted rather than reutilized.

The manuscript is clearly written, the data is of a high technical quality and is well presented, and clearly support the conclusions drawn. These results provide a significant advance in our understanding of RNA metabolism upon nitrogen starvation and broaden our limited perspective of Rny1 function in a manner distinct to its reported role in oxidative stress. The manuscript should be of interest to the wide audience of EMBO J. The only major criticism of the manuscript I have is that the nature and extent of RNA degradation has not been addressed - which RNAs are being degraded and by what degree?

The following point should be addressed upon revision of the manuscript:

1. Based on the abundance of ribosomal RNA in the cell, the authors state that "It was likely that the nucleosides we detected were mostly derived from rRNAs" (line 151, p8). However, it is not clear what fraction of cellular RNA is targeted to the vacuole upon nitrogen starvation or from which RNA species the released nucleosides are generated. The authors should analyse the changes in cellular RNA profile (in particular rRNAs, tRNA and some nuclear RNAs such as specific snRNAs and snoRNAs) upon nitrogen starvation in wild-type and rny1 mutant cells to demonstrate which RNAs are being depleted and to what extent this is occurring.

1st Revision - authors' response 11 October 2014

Referee #1:

The study by Huang et al. deals with starvation-induced RNA catabolism by autophagy. Utilizing metabolome, genetic and biochemical analysis, the authors identify the nucleotidase Pho8 and the ribonuclease Rny1 as the responsible enzymes for vacuolar RNA degradation. Additional data of this manuscript further allowed the identification of the nucleosidases Pnp1 and Urh1, responsible for the following break down into purine and pyrimidine in the cytosol. Furthermore, the manuscript provides evidence that the autophagic core machinery is essential while proteins involved in selective autophagy play a less significant role in this process. Interestingly, also Ubp3 and Bre5, proteins essential for ribophagy, the selective autophagic degradation of ribosomes, were found none essential for this process.

Taken together the data of this manuscript provide convincing evidence for starvation induced autophagic RNA degradation. The authors should address the concerns listed below and provide a more detailed discussion regarding the conflict with the reports on ribophay.

Concerns:

1.A more detailed characterization of the autophagic factors needed for this process should be presented to improve the authors' model.

According to your suggestion, we added a selective autophagy-mutant, *atg32*Δ, specifically defective in mitophagy, to further examine the contribution of selective autophagy to starvation-induced RNA degradation (Figure 2). In combination with EM images showing non-selective bulk autophagy proceeds (Figure 1), all data indicated the fact that generation of nucleosides is not dependent on any factors examined which are specific for selective autophagy. The detailed discussion on the contribution of Atg11 is described below.

*2. Fig.1B: atg11*Δ *cells show a significant decrease in nucleoside levels, suggesting some aspects of selectivity. The authors should therefore discuss this in more details*

Your sceptical opinion on this issue has been very helpful. To further elucidate the specific role of Atg11-involved autophagy, we performed the ALP assay of *atg11*Δ (see Figure 2B) and this mutant showed reduced autophagic activity compared with the wild-type strain, which is considered to directly lead to the low nucleoside levels in *atg11*Δ. Therefore, we believe that the contribution of Atg11-dependent selective autophagy is low compared to non-selective autophagy.

3. Furthermore, this figure should also include the relative intensity of uridine after 3 hours of starvation to show the delayed drop indicated in figure 1 A.

Thank you for your suggestion. We repeated the experiment and extended the sampling duration to 3 hours after starvation (see Figure 2A).

4. Fig.2A: compared to figure 1 the relative intensity of adenosine starts already very high at 0 hours of starvation showing high standard deviation. These data lower the reliability of the assay.

We apologize for the confusing data of adenosine. Accurate measurement of adenosine by LC/MS has always been technically and intrinsically difficult. Furthermore, since 3'-AMP is converted by two-distinct routes, either to adenosine by Pho8 or to 3'-IMP by unknown enzyme protein, the flow of adenosine is indeed fluctuating, so the data are variable from experiment to experiment. We repeated this experiment and data consistent with Figure 1B was successfully obtained (as shown in the revised Figure 2A).

5. Fig2B: The different levels of 5'NMPs and the resulting meaning should be better described in the text.

We agree that 5'-NMP levels are slightly higher in *pho8*Δ cells than those in wild-type and *atg2*Δ cells (Figure 3B). Given that the cytosol contains considerably high levels of 5'-nucleotides and other related compounds such as NAD or UDP-glucose, it is possible that a portion of them is enwrapped into autophagosomes and delivered to the vacuoles, in which they might retained in the absence of Pho8. We mentioned this point in the main text.

6. Fig 3C: testing the effect of atg2Δrny1Δ double knockout would help solidify the conclusion of an autophagic dependent metabolism.

We appreciate your kind advice and accordingly examined *atg2Δrny1Δ* with GR Green upon starvation (Figure 4C). In contrast to the strong fluorescence signals inside vacuoles in *rny1Δ,* no vacuolar stain was found in *atg2Δrny1Δ* either before or after starvation. This result strongly supported our conclusion that the RNA degradation mechanism is autophagy-dependent.

7. Fig.4: the authors should include morphological analysis of bre5Δ and ubp3Δ cells using the Gel Green dye to monitor free RNA in the vacuoles after starvation.

According to your critical suggestion, we performed microscopic analysis, to monitor free RNA in the vacuole, using *ubp3Δrny1Δ* and *bre5Δrny1Δ* cells (Figure 5B). As a result, for both strains the vacuoles were stained with GR green, demonstrating that RNAs are transported to the vacuole in the absence of Ubp3 and Bre5. It indicates that starvation-induced RNA degradation is irrelevant to the function of Ubp3 and Bre5.

8. Fig5: The deviations in the mean values are missing.

Thank you for your kind consideration. In fact (as being described in figure legend) the data in Figure5 (in the revised manuscript, new Figure 6) were calculated by using standard addition method, where the 1 h-point–starvation-samples of wild-type were used as spike-in samples for a standard mixture series of increasing concentrations (see flow chart of the calculation procedures in the Supplemental Experimental Procedures.). Apart from 1 h data, 0 h and 2 h data are obtained as means of duplicates. Technically, standard deviations can only be calculated for data with a replicate of 3 or above. It is not statistically meaningful to calculate the standard deviation for duplicate samples.

9. Fig E2: A negative control e.g. atg2Δ is missing.

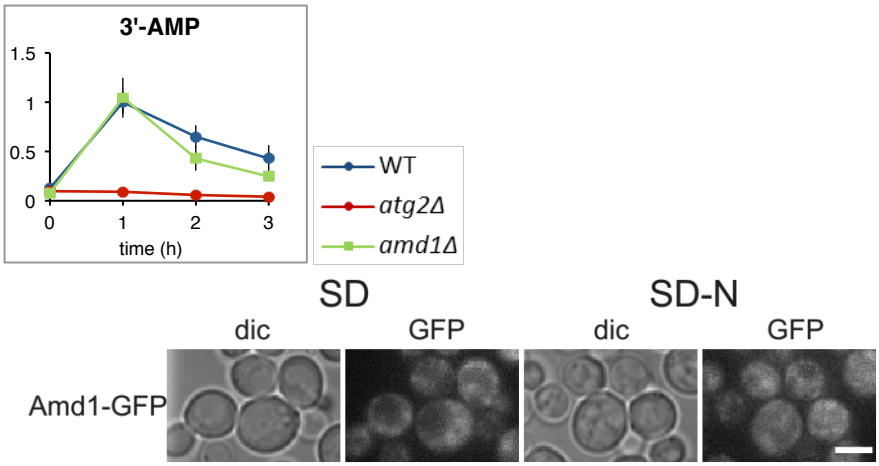
Based on your suggestions, we repeated this experiment with both the positive (WT) and negative (*atg2Δ*) controls and the data have been replaced (see Figure E2A).

10. FigE5: As FigE5 contains mainly the data of figure 5 it should be included in figure 5 and statistical information should be added.

Yes, we understand your point. We greatly appreciate your suggestion. However, we intentionally separated Figure 5 from Figure E5 (in the revised manuscript, new Figure 6, and Figure E5), because it might be easier for readers to understand the distinct arguments we would like to say. In Figure 6 and Figure E5, we simultaneously measured both intracellular and extracellular metabolites of wild-type, *atg2Δ*, and *pho8Δ* cells. For Figure 6, we omitted *pho8Δ* data because in Figure 6 we want to show the apparent autophagy-dependent changes of each nucleoside/nucleobase in and out of the cells. For Figure E5B, we simply wanted to reveal the fact that in the *pho8Δ* cells where intracellular 3'-NMP is highly accumulated, some population of 3'-NMP is exported out of the cells. It implies that vacuolar and plasma membranes possess transporter systems for these metabolites. We hope you would agree that Figure 6 and Figure E5 are kept this way to better serve our purposes.

11. The authors mention Amd1 as a potential deaminase for generation of 3'IMP. This hypothesis should be checked in amd1Δ cells

This suggestion has been really important for making our manuscript more accurate and reliable. According to your suggestion, we performed analysing *amd1*Δ cells. To check the function of Amd1 as a deaminase, we particularly focused on the amount of 3'-AMP in *amd1*Δ cells under starvation, because 3'-IMP level could not be measured due to lack of a high-quality authentic standard. We found that 3'-AMP accumulated to a similar level with that in wild-type cells. Furthermore, we monitored the localization of Amd1-GFP under both growth and starvation conditions, and found that Amd1 was always located exclusively in the cytoplasm but not in the vacuole. Thus, it is unlikely that Amd1 is the responsible vacuolar deaminase for 3'-AMP. For these reasons, we deleted the sentences regarding this content.



12. A more elaborate discussion on the physiological meaning of vacuolar RNA catabolism should be given?

When there are not sufficient nutrients for cells to maintain their minimal essential activities, adjusting their ribosome numbers in order to reduce the rate of protein synthesis can be critical. As described in the discussion, vacuolar ribosomal degradation under nitrogen starvation provides the essential nutrient (amino acids) for their survival, whereas the degradation products of RNA are not re-assimilated, but excreted from the cells. Thus, without an efficient RNA degradation system, RNAs released from the decomposed ribosomes will accumulate in the vacuole to an extremely high level and finally jeopardize the normal enzymatic activities inside the vacuole. Although we have not yet found any specific phenotypes resulted from the incapability of RNA catabolism in yeast, several studies on serious dysfunctions due to the accumulation of rRNA within lysosomes in animals have been reported (Haud *et al.*, 2011; Henneke *et al.*, 2009). The physiological meaning of vacuolar RNA catabolism is undoubtedly an important issue which needs to be further investigated.

Minor

13. Fig E1: Is there any particular reason why this figure is lacking adenosine?

The data of adenosine have been included.

14. Figure E4 and E5 should be exchanged.

Thanks to your critical comment, we just realized that we had an improper citation of Figure E5A, which was somehow redundant and unnecessary and had also put the figures into disorder. Accordingly, we deleted this citation in the context and we believe these figures are in their appropriate order now.

15. Line 399 of discussion should point to the figure 3 to make it easier to follow.

Thank you for your comment and we amended the text accordingly.

Referee #2:

In their manuscript 'Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast', Hanghang et al. present detailed matabolomic studies of autophagy-induced RNA degradation in S. cerevisiae and assay the transformations of the resulting metabolites. In addition, presented data are strengthened by analogous results obtained in S. pombe. The manuscript is well organized, consecutive experiments are introduced in an orderly and logical fashion and the figures are clear and well described. The observation that during nitrogen starvation RNA becomes degraded to nucleosides and bases via 3'-NMPs and the resulting products are further deaminated and excreted from the cells is of major basic biological interest - it is actually surprising that a topic of such importance has never before been this extensively tackled in the autophagy research. The following points need to be further addressed:

1. the authors suggest that non-selective autophagy (as opposed to ribophagy) is responsible for the vacuolar RNA degradation - in this context it would be interesting to see a comparison of degradation kinetics of some ribosomal proteins, chosen rRNA molecules (possibly from both subunits) and other intracellular RNA species (tRNA and mRNA) - this experiment could provide additional evidence that the nitrogen starvation-induced RNA degradation proceeds via non-selective bulk degradation of cytoplasmic constituents

Thank you so much for your complimentary comments.

We evaluated how much and what kind of cellular RNA is targeted to the vacuole via autophagy by directly analysing RNA (Figure E6). The same amount of RNA from wild-type, *atg2*Δ, and *rny1*Δ cells before and after nitrogen starvation was examined by northern-blot and quantitative RT-PCR (qRT-PCR). Based on the assumption that the amount of small nuclear non-coding RNAs such as snRNA and snoRNA is not affected by autophagy, we employed them as internal references. By doing so, we observed decreases in both 25S and 18S RNAs as well as several tRNAs in wild-type cells compared to those in *atg2*Δ and *rny1*Δ cells under starvation conditions by both northern blot and qRT-PCR analyses (Figure E6A-C). We understand that it is difficult to quantitate the accurate amount of RNAs and kinetics of their decrease by these conventional methods, but the trend shown in these results has undoubtedly demonstrated that cytoplasmic RNAs are degraded under starvation via autophagy. By contrast, as shown in this study, metabolome analysis quantitating the increase of

degradation products such as nucleosides, is far more sensitive and accurate to define bulk RNA degradation via autophagy. We believe this fully supported our conclusion that non-selective bulk degradation to be the primary pathway for starvation-induced RNA degradation.

For RNA degradation, it is interesting to know if there are some specific RNA species of tRNA, mRNA, or other cytoplasmic RNAs selectively degraded by autophagy. So far we did not find any selectivity in autophagy-mediated RNA degradation. (Figure E6B and E6C). However, global analysis such as RNA-seq technique might reveal a more accurate assessment of changes in RNA species that are regulated by autophagy, if any.

2. As an addendum to the presented data obtained under nitrogen-starvation conditions, additional autophagic stimuli (different types of starvation or pharmacologic treatment) could be briefly tested (here analysis of nucleoside accumulation kinetics would suffice) - this would give a broader picture of autophagic RNA degradation and would be of great general interest

According to your suggestions, we newly performed the time-course experiments with two other different stimuli: carbon-starvation and rapamycin treatment. Increases in the levels of nucleosides were successfully detected in an autophagy-dependent manner (see Figure 1C-D). The data indicates the generality of RNA degradation regulated by autophagy.

other remarks:

3. in lines 133 and 164 the word 'significantly' is used in relation to results on which no statistical significance tests were performed - please add the missing statistics.

Thank you for helping us make the description in the manuscript more accurate. We have rephrased this part.

4. for data represented in Fig. E3B, p values should be included

p values have been included as suggested.

5. there's a marked discrepancy between adenosine levels depicted in Fig. 1B and 2A - I believe that it can be at least partially explained by the generally low levels of this metabolite in the cells (as shown in Fig. 5B) but still some word of clarification would be beneficial in order to avoid confusing the readers

We apologize for the confusing data of adenosine. Accurate measurement of adenosine by LC/MS has always been technically and intrinsically difficult. In addition, since by two-distinct routes 3'-AMP is converted: either to adenosine by Pho8 or to 3'-IMP by an unknown protein, the flow of adenosine is indeed fluctuating, so the data are slightly variable from experiment to experiment. We repeated this experiment and data consistent with Figure 1B was successfully obtained (see Figure 2A).

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Rny1 and Pho8, further metabolized to its bases in the cytosol and ultimately excreted rather than reutilized.

The manuscript is clearly written, the data is of a high technical quality and is well presented, and clearly support the conclusions drawn. These results provide a significant advance in our understanding of RNA metabolism upon nitrogen starvation and broaden our limited perspective of Rny1 function in a manner distinct to its reported role in oxidative stress. The manuscript should be of interest to the wide audience of EMBO J. The only major criticism of the manuscript I have is that the nature and extent of RNA degradation has not been addressed - which RNAs are being degraded and by what degree?

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We understand your concern. We performed experiments as you suggested, and added data and discussed in the main text as described below.

From the nucleobases excreted to the medium, we could estimate that a 3-4 % of RNAs were catabolized per hour under nitrogen starvation conditions. About 80-85 % of total cellular RNA is rRNAs and 10-15 % is tRNA, whereas mRNA and other non-coding RNAs are minor. Therefore most RNA degraded under starvation observed must be rRNA sequestered into autophagosomes, which is supported by the electron micrograph shown in Figure 1A. We tried to evaluate how much and what kind of cellular RNA is targeted to the vacuole via autophagy by directly analysing RNA (Figure E6). The same amount of RNA from wild-type, *atg2*Δ, and *rny1*Δ cells before and after nitrogen starvation was examined by northern-blot and quantitative RT-PCR (qRT-PCR). Based on the assumption that the amount of small nuclear non-coding RNAs such as snRNA and snoRNA is not affected by autophagy, we employed them as internal references. By doing so, we observed decreases in both 25S and 18S RNAs as well as several tRNAs in wild-type cells compared to those in *atg2*Δ and *rny1*Δ cells under starvation conditions by both northern blot and qRT-PCR analyses (Figure E6A-C). However, we understand that it is quite difficult to quantitate the accurate amount of RNAs and kinetics of their decrease by these conventional methods. By contrast, as shown in this study, metabolome analysis quantitating the increase of degradation products such as nucleosides, is far more sensitive and accurate to define bulk RNA degradation via autophagy.

For RNA degradation, it is interesting to know if there are some specific RNA species of tRNA, mRNA, or other cytoplasmic RNAs selectively degraded by autophagy. So far we did not find any selectivity in autophagy-mediated RNA degradation. (Figure E6B and E6C). However, global analysis such as RNA-seq technique might reveal a more comprehensive assessment of changes in RNA species that are regulated by autophagy, if any.

Acceptance 28 October 2014

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been adequately addressed and I am therefore happy to inform you that your manuscript has been accepted for publication with us. However, before we can proceed to transfer the manuscript for production I have to ask you to address the following minor editorial points:

-> Could you please follow the suggestion by referee #3 and introduce the data on RNA expression changes following nitrogen starvation in the results section of the manuscript?

-> Could you please also remove the reference to unpublished data on p.16? I am afraid our journal policies do not allow references to 'data not shown' and I would therefore ask you to either include this data in a supplemental figure or to rephrase the sentence in question.

An amended manuscript text file can be sent to me by email and we will then upload it in house.

REFEREE REPORTS:

Referee #1:

The authors addressed my comments and the manuscript now meets the scientific merit of EMBO J

Referee #3:

The authors have responded to my comments appropriately and the manuscript is, in my opinion, suitable for publication in EMBO J.

The northern and RT-PCR data addressing changes in RNA levels upon nitrogen starvation do not reveal Atg2-dependent effects on specific RNAs. However, this does not impact on the main findings of the paper. This new data (Figure E6) should be shown in the Results section, however, and not incorporated in the Discussion.

