THOC4 regulates energy homeostasis by stabilizing *TFEB* mRNA during prolonged starvation

Toshiharu Fujita, Sayaka Kubo, Tatsuya Shioda, Ayaka Tokumura, Satoshi Minami, Megumi Tsuchiya, Yoshitaka Isaka, Hidesato Ogawa, Maho Hamasaki, Li Yu, Tamotsu Yoshimori and Shuhei Nakamura
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Original submission

First decision letter

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MS TITLE: THOC4 regulates energy homeostasis by stabilizing TFEB mRNA

AUTHORS: Toshiharu Fujita, Sayaka Kubo, Tatsuya Shioda, Satoshi Minami, Megumi Tsuchiya, Hidesato Ogawa, Maho Hamasaki, Li Yu, Yoshitaka Isaka, Tamotsu Yoshimori, and Shuhei Nakamura
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to:

https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access)

As you will see from their reports, all three reviewers raise a number of substantial criticisms that prevent me from accepting your paper for publication.

I am very sorry to give you such disappointing news, but we are currently under great pressure for space and it takes a very enthusiastic recommendation by the referees for a manuscript to be accepted.

I do hope you find the comments of the reviewers helpful in allowing you to revise the manuscript for submission elsewhere, and many thanks for sending your work to Journal of Cell Science.

Reviewer 1

Advance summary and potential significance to field

TFEB acts as a master transcription factor controlling the expression of a plethora of genes involved in the autophagy-lysosome pathway. TFEB undergoes cytoplasmic-nuclear shuttling, a process controlled by its phosphorylation mediated by the nutrient sensor mTORC1. In this study, Nukamura and colleagues uncovered a novel mechanism in regulating TFEB activity. The authors showed that the mRNA-binding protein THOC4 functions as a TFEB regulator. Knocking down THOC4 activity decreases the TFEB protein level. THOC4 binds to and stabilizes TFEB mRNA. The authors further showed that this regulatory mechanism is conserved in C. elegans. However, the result showing that a reduction in TFEB level by THOC4 depletion causes an increase in autophagic flux is counterintuitive. This needs to be addressed. Otherwise, the authors cannot make the conclusion that THOC4 is involved in autophagy regulation via TFEB.

Comments for the author

Specific concerns:

1. By using the mRFP-GFP tandem LC3 reporter, the authors showed that siRNA-mediated THOC4 knockdown increases the population of GFP-/RFP+ autolysosomes.
Based on this observation, they suggested that the autophagic flux is increased by THOC4 knockdown. Other assays such as measurement of levels of p62 and LC3 should be performed to consolidate this conclusion. The RFP-positive autolysosomes may be acidified but non-degradative. EM analysis can also be performed to directly visualize autophagic structures.
2. The authors claimed that THOC4 depletion increases autophagy activity by repressing mTOR activity based on a reduction in the level of phosphorylated S6 kinase. mTOR inactivation also promotes autophagosome formation. The autophagic flux in THOC4 KD cells should be thoroughly analyzed using a series of reporters for autophagic structures at different stages.
3. The level of TFEB protein is dramatically reduced in THOC4 KD cells.
Counterintuitively, autophagy activity is increased. This issue needs to be resolved. The autophagy activity was examined in THOC4 knockdown cells. The autophagy phenotype should be determined in THOC4 knockout cells.
4.The authors show that levels of HLH-30::GFP are reduced by depleting THOC4 in C. elegans. This could be caused by a non-selective effect of THOC4 on expression of a transgene. Endogenous hlh-30 mRNA levels need be examined to confirm the statement.
4. Page 3, the authors stated that “Expression of TFEB itself was also upregulated by starvation, but the mechanism responsible for this upregulation remains unclear”. Previous studies have demonstrated that TFEB exhibits an auto-activation property.

Reviewer 2

Advance summary and potential significance to field

In this study, Fujita et al. found that the expression of TFEB, which serves as a master transcription factor for the regulation of lysosome biogenesis autophagy, and lipid metabolism, is maintained by the RNA-binding protein THOC4. Specifically, the authors showed that THOC4 knockdown decreased TFEB at both mRNA and protein levels. The authors further provided the results suggesting that THOC4 protects TFEB mRNA from degradation probably by stabilizing its poly A tail. Consistently, THOC4 knockdown affected autophagic activity and lipid metabolism in mammalian and worm cells. Thus, this study discovers a new mechanism that ensures the expression of TFEB. However, this reviewer finds the present work is too preliminary to allow the authors to convincingly made the conclusion.

Comments for the author

Major points:
(1) The authors should perform rescue experiments to exclude possible off-target effects of siRNA.

(2) Fig. 2C, D: As all the mRNA examined were pulled down with THOC4 to a similar extent, the authors should add a control RNA that does not associate with THOC4.

(3) The authors showed that THOC4 knockdown differently affected mRNA levels of several transcription factors, although THOC4 binds to these mRNAs to a similar extent. The authors should provide more mechanistic insights into how THOC4 preferentially stabilizes TFEB mRNA.

(4) The authors have not provided any evidence that THOC4/aly knockdown affected lipid metabolism through TFEB/hlh-30 downregulation. Is the accumulation of lipid droplets cancelled if TFEB/hlh-30 expression was increased to a normal level (not overexpression)?

(5) Fig. 3B, C: The authors should provide more convincing results or perform another assay to conclude that THOC4 is important for autophagic flux. The present results may show decreased LC3 expression rather than autophagic flux.

(6) Throughout the manuscript, the authors stated that THOC4 "regulates" TFEB expression, but there is no evidence supporting this statement; what the present results suggest is that THOC4 is important to maintain TFEB mRNA levels. It would be interesting to examine whether the effects of THOC4 knockdown on TFEB mRNA levels and/or THOC4 association with TFEB mRNA differ among nutrient replete conditions and early and prolonged starvation conditions.

(7) Fig. 3H, I: The results show that THOC4 knockdown resulted in lipid droplet accumulation under nutrient replete conditions as much as starvation conditions. These results suggest that lipid droplet accumulation is not related to the role for THOC4 during prolonged starvation.

Minor points:
(1) In Fig. S1A and B, the fluorescence microcopy images seem not to be representatives of the quantification results.

(2) In Fig. 1A, B, G, as opposed to the authors' statement, THOC4 knockdown also significantly decreased TFE3 (especially in the immunoblotting image shown in Fig. 1A). Therefore, these results do not support the conclusion that THOC4 preferentially regulates TFEB among these transcription factors.

(3) Figs. 1E and 3B: What do these single bands represent, LC3-I or LC3-II?

(4) I was confused about the results shown in Fig. S1. Why did THOC4 knockdown affect autophagy in panels A-D but not in panel H?

(5) Line 145: "Poly-ubiquitin" should be "poly-ubiquitylated proteins".

(6) Line 173: "TFEB mRNA the presence of" should be "TFEB mRNA in the presence of".

Reviewer 3

Advance summary and potential significance to field

In the manuscript titled “THOC4 regulates energy homeostasis by stabilizing TFEB mRNA”, Fujita and colleagues summarize their finding that TFEB mRNA is directly stabilized by THOC4 binding to mRNA. They found siRNA KD of THOC4 increased the autolysosome population, inhibited mTOR activity, and reduced the level of TFEB protein that is MG132 and BafA1 insensitive, and the overall mRNA level of TFEB decreased drastically.

Comments for the author

There are several points that do not make sense to the reviewer.

1. It is not clear how THOC4 binds to TFEB mRNA. Presumably, one possibility is that TFEB mRNA is generally short-lived, thus by breaking the efficient trafficking of mRNA into the cytosol the overall

TFEB mRNA and protein level both will go down. Especially more confounding because the previous results on METTL3 and m6A modification of TFEB mRNA (Song 2019 Autophag) already showed TFEB mRNA stability modulation through mRNA modification.

2. How does the exogenous expression of TFEB, which would likely have no 5'UTR and 3'UTR, have reduced protein level? Does the mRNA sequence of exogenously expressed TFEB match the original gene sequence?

3. It's not clear how THOC4 specifically induce TFEB mRNA stability modulation. The above result indicates that, if the mRNA ORF sequence matches, that the regulation is NOT likely due to 5' or 3'UTR but rather the ORF sequence itself. Have you tried to perform sense mutations to see if these results remain true?

4. If the answer to question 2 is no, it doesn't seem like the level of regulation is at the mRNA by stabilization of mRNA but rather seems to be more complicated regulation, potentially with modulating other cis-acting elements.

Author rebuttal letter

Dear Prof. Dr. Lippincott-Schwartz,

I am Shuhei Nakamura from Prof. Yoshimori's laboratory at Osaka University.

Thank you so much for your kind consideration of our previous manuscript in Journal of Cell Science (MS ID#:JOCES/2020/248203), and giving us the the insightful comments from the reviewers. According to the reviewers' comments, we conducted lines of experiments and made a significant progress in our study.

Especially, the reviewer asked how THOC4 knockdown activates autophagy in the absence of TFEB and we got solid evidences regarding TFEB independent autophagy activation by THOC4 knockdown (EM analysis, WB, IHC etc).

In addition, although we could not fully understand how THOC4 regulates TFEB stability, the experiment using the deletion constructs indicated at least TFEB coding sequence but not UTR is essential for TFEB stability and the mechanism seems independent from METTL3 function which one of reviewers asked.

I believe that the revised manuscript will provide a novel insight into TFEB regulation at level of mRNA and also indicate the presence of TFEB independent autopahgy induction process as revealed by our study focused on THOC4.

If you allow us to resubmit the manuscript, could you please tell us how to proceed?
Again, we appreciate your kind consideration of our manuscript. We look forward to hearing from you, soon.

Rebuttal response letter

MS ID#: JOCES/2020/248203

MS TITLE: THOC4 regulates energy homeostasis by stabilizing TFEB mRNA

AUTHORS: Toshiharu Fujita, Sayaka Kubo, Tatsuya Shioda, Satoshi Minami, Megumi Tsuchiya, Hidesato Ogawa, Maho Hamasaki, Li Yu, Yoshitaka Isaka, Tamotsu Yoshimori, and Shuhei Nakamura

Dear Dr. Nakamura,

Thank you for your recent letter. I understand how disappointed you must feel.

Given the opinions stated by the reviewers, I had no choice but to reject the paper.

However, we are always willing to give authors the chance to defend their manuscripts. In the light of the comments you make in your letter, I have decided to proceed as follows. I would be happy for you to submit a revised version of your manuscript that deals as far as possible with the points raised by the reviewers, together with a detailed rebuttal of any other matter that cannot be settled in the manuscript itself. I will then send the revised version and the rebuttal back to the reviewers. If they are convinced by your arguments, then we would be happy to accept the manuscript for publication.

First revision

Author response to reviewers' comments

Reviewer's comments;

Reviewer #1:
Advance Summary and Potential Significance to Field:
TFEB acts as a master transcription factor controlling the expression of a plethora of genes involved in the autophagy-lysosome pathway. TFEB undergoes cytoplasmic-nuclear shuttling, a process controlled by its phosphorylation mediated by the nutrient sensor mTORC1. In this study, Nakamura and colleagues uncovered a novel mechanism in regulating TFEB activity. The authors showed that the mRNA-binding protein THOC4 functions as a TFEB regulator. Knocking down THOC4 activity decreases the TFEB protein level. THOC4 binds to and stabilizes TFEB mRNA. The authors further showed that this regulatory mechanism is conserved in C. elegans. However, the result showing that a reduction in TFEB level by THOC4 depletion causes an increase in autophagic flux is counterintuitive. This needs to be addressed. Otherwise, the authors cannot make the conclusion that THOC4 is involved in autophagy regulation via TFEB.

Response
First of all, we thank the reviewer for investing tremendous efforts and times to review our paper and providing very valuable comments. As we mentioned in the main text, regulation of TFEB by THOC4 could be important during prolonged starvation. In the nutrient-rich condition, autophagic activity was elevated by THOC4 knockdown (Fig.S1) despite the dramatic decrease of TFEB protein. Although TFEB is known as a master transcriptional regulator of autophagy, how this is achieved? As shown in the main text, we conducted series of experiments to confirm THOC4 knockdown indeed activated autophagy and this was independent of TFEB function. So far, as a possible explanation of autophagy upregulation, mTOR activity was decreased by THOC4 depletion as shown by decreased phosphorylation of mTOR direct targets p70-S6K (Fig.S3 A,B). This phenotype was also observed in TFEB KO cells (Fig.S3 C,D). Furthermore, we found that one of the mTOR upstream inhibitor TSC2 was transcriptionally upregulated upon THOC4 knockdown (Fig.S3 I) which was also the case in TFEB KO cells (Fig.S3 J). Therefore, THOC4 could regulates autophagy via TFEB-independent inhibition of mTOR.

In downstream of mTOR, it is reported that the prevention of UVRAG Ser498 phosphorylation facilitates autophagosome maturation (Kim et al., *Mol Cell*, 2015). We found that the level of UVRAG phosphorylation at Ser498 was significantly decreased upon THOC4 knockdown both in WT and TFEB KO cells. Considering these facts, THOC4 knockdown may activate autophagy by inhibiting UVRAG phosphorylation by mTOR.

qPCR experiment also showed the transcriptional upregulation of many autophagy related genes by THOC4 knockdown and it was also seen in TFEB KO cells (Fig.S4 A,B). These results suggest that THOC4 may transcriptionally regulates autophagy independent of TFEB. Also to note, these upregulated genes were almost back to normal level after 12 h prolonged starvation (Fig.S4 C), possibly explaining the predominated TFEB function during prolonged starvation.

Specific concerns:
1. By using the mRFP-GFP tandem LC3 reporter, the authors showed that siRNA-mediated THOC4 knockdown increases the population of GFP-/RFP+ autolysosomes. Based on this observation, they suggested that the autophagic flux is increased by THOC4 knockdown. Other assays such as measurement of levels of p62 and LC3 should be performed to consolidate this conclusion. The RFP positive autolysosomes may be acidified but non-degradative. EM analysis can also be performed to directly visualize autophagic structures.

Response
We thank the reviewer for pointing this out. We checked the p62 protein level as you suggested. p62 was significantly decreased by THOC4 knockdown (Fig.S1 I,J and K,L) indicating the elevated autophagic activity. This decrease was also observed in TFEB KO cell (Fig.S1 M,N).
We also conducted a LC3 flux assay and found that LC3 flux was increased by THOC4 knockdown in the nutrient-rich condition (Fig.S1 G,H) while it was decreased during prolonged starvation (Fig. 4 C,D).
We also conducted the EM analysis to visualize the autophagic vacuole as you suggested (Fig. S2). Then we confirmed the accumulation of autophagosome like double-membrane structures in THOC4 knockdown cells, especially in nutrient rich condition. In 2 h starved cells, no such big difference was observed comparing to siRNA control, and it is consistent with LC3 flux assay (Fig. S2 G,H).

2. The authors claimed that THOC4 depletion increases autophagy activity by repressing mTOR activity based on a reduction in the level of phosphorylated S6 kinase. mTOR inactivation also promotes autophagosome formation. The autophagic flux in THOC4 KD cells should be thoroughly analyzed using a series of reporters for autophagic structures at different stages.

Response
The phosphorylation rate of the downstream target of mTOR ULK1 was not significantly changed as we evaluate the phosphorylation rate at Ser757 (data not shown). However, phosphorylation of UVRAG at Ser498, which is also known as a mTOR target, and reported to negatively regulates autophagosome maturation (Kim et al., *Mol Cell*, 2015) was decreased by THOC4 knockdown independently of TFEB (Fig.S3 G,H). As we showed in (Fig. S1 C,D and E,F), the number of LC3 dots and GFP-Atg5 dots were increased by THOC4 knockdown. Thank you.
3. The level of TFEB protein is dramatically reduced in THOC4 KD cells. Counterintuitively, autophagy activity is increased. This issue needs to be resolved. The autophagy activity was examined in THOC4 knockdown cells. The autophagy phenotype should be determined in THOC4 knockout cells.

Response
We showed the activation of autophagy upon THOC4 depletion with several assays, tf-LC3 assay, LC3 and GFP-Atg5 dot formation, LC3 flux assay and decreased p62 protein (Fig.S1). As a possible mechanism of autophagy activation, THOC4 knockdown transcriptionally upregulates TSC2 (Fig.S3 I) and inhibits mTOR activity (Fig.S3 A,B). As a consequence, UVRAG Ser498 phosphorylation was decreased (Fig.S3 G,H). Importantly, these phenotypes were also found in TFEB KO cells, indicating that THOC4 could negatively regulate autophagy in a TFEB independent manner.
Although we could not obtain THOC4 KO cells during the limited period, autophagy upregulation by THOC4 knockdown was confirmed by using several different cell types, HeLa, MEF, HEK cells. While almost all experiments were performed using HeLa cells, the upregulation of LC3 flux assay was also confirmed using MEF cells (Fig.S1 G,H). The decreased p62 protein indicating the activation of autophagy by THOC4 knockdown observed in HeLa cells (Fig.S1 K,L) was also the case in HEK293 cells (Fig.S1 I,J).

4. The authors show that levels of HLH-30::GFP are reduced by depleting THOC4 in C. elegans. This could be caused by a non-selective effect of THOC4 on expression of a transgene. Endogenous hlh-30 mRNA levels need be examined to confirm the statement.

Response
We conducted a qPCR experiment and found that HLH-30 transcripts were not altered in each aly-1, 2, 3 KO worm. Due to the difficulty of the generation of triple KO worm, because aly-1, 2 and 3 are all in the same chromosome IV, we knock-downed aly-2, 3 in aly-1 KO worm background and found that endogenous hlh-30 transcripts were significantly decreased (Fig.4 G). Thank you.

5. Page 3, the authors stated that "Expression of TFEB itself was also upregulated by starvation, but the mechanism responsible for this upregulation remains unclear". Previous studies have demonstrated that TFEB exhibits an auto-activation property.

Response
We thank the reviewer for pointing this out. We checked the possibility that the upregulation of TFEB mRNA during prolonged starvation (Fig.4 A) could be due to the autoregulation by TFEB itself. TFEB promotes its transcription by binding its promotor sequence known as CLEAR motif. When we check with the exogenously overexpressed TFEB-mNG which lacks own promoter including CLEAR motif and UTRs, the expression was also increased during prolonged starvation (Fig. 4B). This result suggests presence of the additional upregulation mechanism in addition to auto-activation by TFEB itself depending on the cell type and/or context.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this study, Fujita et al. found that the expression of TFEB, which serves as a master transcription factor for the regulation of lysosome biogenesis, autophagy, and lipid metabolism, is maintained by the RNA-binding protein THOC4. Specifically, the authors showed that THOC4 knockdown decreased TFEB at both mRNA and protein levels. The authors further provided the results suggesting that THOC4 protects TFEB mRNA from degradation probably by stabilizing its poly A tail. Consistently, THOC4 knockdown affected autophagic activity and lipid metabolism in mammalian and worm cells. Thus, this study discovers a new mechanism that ensures the expression of TFEB. However, this reviewer finds the present work is too preliminary to allow the authors to convincingly made the conclusion.

Reviewer 2 Comments for the Author:

Major points:
(1) The authors should perform rescue experiments to exclude possible off-target effects of siRNA.

Response
Thank you very much for your valuable comments about our work. Since we could not obtain KO cells in this period for the rescue experiment, to exclude the possible off-target effects of siRNA against THOC4, we tried with two other additional siRNAs. Treatment with siTHOC4 #2 and #3 also significantly decreased the TFEB protein (Fig.S5 A,B). Moreover, autophagy induction by THOC4 knockdown was also confirmed in HEK and MEF cells in addition to Hela cells, largely ruling out the off-target effect (Fig.S1 G-J).

(2) Fig. 2C, D: As all the mRNA examined were pulled down with THOC4 to a similar extent, the authors should add a control RNA that does not associate with THOC4.

Response
Thank you very much to point this out. We tried to found possible control that does not bind to THOC4, but unfortunately, we could not find any negative control in our revision period. THOC4 is reported to bind many mRNA to facilitate its nuclear export. So THOC4 may associate with amounts of mRNAs to similar extent.
We therefore our experimental set up by confirming that the band detected in our RIP experiment was not found in absence of reverse transcriptase (Fig. shown below), reflecting the actual binding to TFEB mRNA by THOC4.

Note: We have removed unpublished data provided for the referees in confidence.

(3) The authors showed that THOC4 knockdown differently affected mRNA levels of several transcription factors, although THOC4 binds to these mRNAs to a similar extent. The authors should provide more mechanistic insights into how THOC4 preferentially stabilizes TFEB mRNA.

Exogenously expressed FLAG-TFEB and stably expressed mNG-TFEB, which do not have the original UTRs, were also dramatically decreased by THOC4 knockdown (Fig.2 B,C). During prolonged starvation, THOC4 and also TFEB expression was enhanced at mRNA level (Fig.3 A) presumably due to increased mRNA stability conferred by bound THOC4. This upregulation was also the case with exogenously expressed mNG-TFEB (Fig. 4B).

These results indicated that THOC4 may bind to TFEB mRNA at somewhere in the coding region. As you pointed out, THOC4 binds to mRNAs of other transcription factors to a similar extent (Fig.3 A,B) but mRNA stability of especially MITF was not largely affected by THOC4 knockdown (Fig.3 C,D). Since these differences in stability are thought to be due to differences in coding region sequences, we created multiple mutants that deletion in locations other than the common main domain, and examine whether they are affected by THOC4 KD. However, THOC4 KD reduced expression in all mutants tested (Fig. shown below). Maybe we have to look at higher-order structures instead of a single sequence and would like to address more.

Note: We have removed unpublished data provided for the referees in confidence.

(4) The authors have not provided any evidence that THOC4/aly knockdown affected lipid metabolism through TFEB/hlh-30 downregulation. Is the accumulation of lipid droplets cancelled if TFEB/hlh-30 expression was increased to a normal level (not overexpression)?

Response
Thank you very much for your comment. We also confirmed the effect of HLH-30 loss for lipid accumulation using *hlh-30* RNAi. Since THOC4 affects TFEB/HLH-30 through the coding region based on our current findings, it is technically difficult to revert the TFEB expression level to the normal level upon THOC4 knockdown. Alternatively, we showed in *C. elegans* that accumulated lipid in aly KO starved worms was not further accumulated by *hlh-30* RNAi treatment (Fig.5 E,F) indicating that the lipid accumulation in aly mutant could be due to HLH-30 loss.

(5) Fig. 3B, C: The authors should provide more convincing results or perform another assay to conclude that THOC4 is important for autophagic flux. The present results may show decreased LC3 expression rather than autophagic flux.

Response
Thank you very much for point that out. We are confident about our results that the LC3 flux assay from 3 independent experiments (difference between amounts of LC3 II with Bafilomycin and one without Bafilomycin) clearly showed the statistically significant decrease of autophagic flux by THOC4 knockdown during the prolong starvation. In addition, we found that the upregulation of several autophagy genes observed during the nutrient rich condition was not evident during the long-term starvation (Fig.S4 C). These data suggest THOC4 knockdown impaired autophagic flux during the long-term starvation. Regarding the LC3 expression level, we confirmed that LC3 expression was not significantly changed upon THOC4 depletion during prolonged starvation by qPCR (Fig.S4 C). Thank you.

(6) Throughout the manuscript, the authors stated that THOC4 "regulates" TFEB expression, but there is no evidence supporting this statement; what the present results suggest is that THOC4 is important to maintain TFEB mRNA levels. It would be interesting to examine whether the effects of THOC4 knockdown on TFEB mRNA levels and/or THOC4 association with TFEB mRNA differ among nutrient replete conditions and early and prolonged starvation conditions.

Response
Thank you for pointing this out. We tried to compare the levels of association of THOC4 with TFEB mRNA. However, at least in our experimental condition, the levels of association seemed not to be different in prolonged starvation (Fig. shown below). It could be possible that THOC4 recruits

additional components depending on the contexts and this could affect the stability of TFEB. We would like to address this point in our future study by conducting the comprehensive THOC4 and TFEB interactome and subsequent functional analysis. Thank you for your valuable comments and suggestions.

Note: We have removed unpublished data provided for the referees in confidence.

(7) Fig. 3H, I: The results show that THOC4 knockdown resulted in lipid droplet accumulation under nutrient replete conditions as much as starvation conditions. These results suggest that lipid droplet accumulation is not related to the role for THOC4 during prolonged starvation.

Response
We used kidney proximal tubular cells (PTCs) for lipophagy assay as reported (Minami et al., *Autophagy*, 2017). In this assay, cells were first treated with 250 μM oleic acid for 12hrs and then incubated with DMEM or starvation medium without oleic acid for 24hrs to measure Lipid Droplets (LDs). It could be possible that Oleic acid treatment and subsequent wash off with DMEM induces starvation like stress. To avoid confusion, we changed the label in the figure from 'nutrient' to DMEM and 'starvation' to EBSS, respectively. Although we did not show the results but for *C.elegans*, Oil Red O positive area was not different in nutrient-rich condition in aly KO worms compared to wt, confirming the starvation dependent roles.

Minor points:
(1) In Fig. S1A and B, the fluorescence microcopy images seem not to be representatives of the quantification results.

Response
We think the picture in the figure is the representative one.
We show the picture with more cells (Fig. shown below). Some cells still have GFP intensity, may be because the knockdown efficiency was low. We think that is why the total difference was small in the quantification result.

Note: We have removed unpublished data provided for the referees in confidence.

(2) In Fig. 1A, B, G, as opposed to the authors' statement, THOC4 knockdown also significantly decreased TFE3 (especially in the immunoblotting image shown in Fig. 1A). Therefore, these results do not support the conclusion that THOC4 preferentially regulates TFEB among these transcription factors.

Response
We thank the reviewer for pointing this out. We rephrased "preferentially" to "mainly" in the text.

(3) Figs. 1E and 3B: What do these single bands represent, LC3-I or LC3-II?
Response
We thank the reviewer for pointing this out. We marked the band as LC3-ll.

(4) I was confused about the results shown in Fig. S1. Why did THOC4 knockdown affect autophagy in panels A-D but not in panel H?
Response
Sorry for confusing you. We removed panel H from Fig.S1 and elevated LC3 flux is shown in panel G and H.

(5) Line 145: "Poly-ubiquitin" should be "poly-ubiquitylated proteins".

(6) Line 173: "TFEB mRNA the presence of" should be "TFEB mRNA in the presence of".

Response
Thank you very much for pointing these out. These were rewritten as pointed out.

Reviewer 3 Advance Summary and Potential Significance to Field:
In the manuscript titled "THOC4 regulates energy homeostasis by stabilizing TFEB mRNA", Fujita

and colleagues summarize their finding that TFEB mRNA is directly stabilized by THOC4 binding to mRNA. They found siRNA KD of THOC4 increased the autolysosome population, inhibited mTOR activity, and reduced the level of TFEB protein that is MG132 and BafA1 insensitive, and the overall mRNA level of TFEB decreased drastically.

1. It is not clear how THOC4 binds to TFEB mRNA. Presumably, one possibility is that TFEB mRNA is generally short-lived, thus by breaking the efficient trafficking of mRNA into the cytosol the overall TFEB mRNA and protein level both will go down. Especially more confounding because the previous results on METTL3 and m6A modification of TFEB mRNA (Song 2019 Autophagy) already showed TFEB mRNA stability modulation through mRNA modification.

Response
Thank you very much for pointing this out.
Exogenously expressed FLAG-TFEB and stably expressed mNG-TFEB, which do not have the original UTRs, were also dramatically decreased by THOC4 knockdown (Fig.2 B,C). During prolonged starvation, THOC4 and also TFEB expression was enhanced at mRNA level (Fig.4 A) presumably due to increased mRNA stability conferred by bound THOC4. This upregulation was also the case with exogenously expressed mNG-TFEB (Fig. 4B).
These finding may suggest that THOC4 binds to the ORF of TFEB mRNA.
We compared mRNA stability between TFEB and other bHLH transcription factor, HES5 with/without THOC4 knockdown after actinomycin D treatment. We found that Hes5 is generally short lived and decreased to same extent both in control and THOC4 knockdown cells. On the other hand, TFEB is relatively stable compared to HES5 and THOC4 knockdown clearly decreased TFEB mRNA levels. These results suggest that the general mRNA stability is not main cause of TFEB downregulation by THOC4 knockdown (Fig. shown below).

Note: We have removed unpublished data provided for the referees in confidence.

As you mentioned, there is a report showing that METTL3 modulates TFEB mRNA stability by m^6A methylation. They showed that RNA methylation is found in 3'-UTR, however THOC4 should bind to ORF as we mentioned above. So, we think the regulation could be different. We could not see the upregulation of TFEB by METTL3 knockdown in our HeLa cell probably due to different context. But importantly the dramatic decrease of TFEB was still observed by METTL3 and THOC4 double knockdown (Fig. shown below), indicating that METTL3 and THOC4 do not work in the same pathway.

Note: We have removed unpublished data provided for the referees in confidence.

2. How does the exogenous expression of TFEB, which would likely have no 5'UTR and 3'UTR, have reduced protein level? Does the mRNA sequence of exogenously expressed TFEB match the original gene sequence?

Response
Yes, the exogenous TFEB coding sequence is matched with the original and is activated upon starvation or lysosomal damage as we have shown recently (Nakamura et al., Nat Cell Biol, 2020). The construct was originally purchase from Addgene (#38119) and the sequence was confirmed by ourself. As we discussed above, we think THOC4 may bind to the ORF of TFEB mRNA to stabilize it.

3. It's not clear how THOC4 specifically induce TFEB mRNA stability modulation. The above result indicates that, if the mRNA ORF sequence matches, that the regulation is NOT likely due to 5' or 3'UTR but rather the ORF sequence itself. Have you tried to perform sense mutations to see if these results remain true?

Response
Thank you very much for pointing that out. We think the difference of ORF sequence confer the different stabilizing effect by THOC4. Since MITF mRNA stability was not largely altered by THOC4 knockdown (Fig.3D), we generated some truncate mutants of TFEB with the sequence different from that of MITF (Fig. shown below). However, all mutants we tested were still decreased by THOC4 knockdown. Two or more domains may need to be truncated at the same time. We would

like to address how THOC4 preferentially affect TFEB stability through the comprehensive THOC4 and TFEB interactome and subsequent detailed functional analysis in our future study. Thank you.

Note: We have removed unpublished data provided for the referees in confidence.

4. If the answer to question 2 is no, it doesn't seem like the level of regulation is at the mRNA by stabilization of mRNA but rather seems to be more complicated regulation, potentially with modulating other cis-acting elements.

Response
The answer could be yes in the question2, exogenously expressed TFEB match the original gene sequence in its ORF. But there still has possibility that the regulation could be more complicated, and some other RNA binding proteins could be involved in the regulation. We would like to address these points further in our upcoming study.

Second decision letter

MS ID#: JOCES/2020/248203

MS TITLE: THOC4 regulates energy homeostasis by stabilizing TFEB mRNA

AUTHORS: Toshiharu Fujita, Sayaka Kubo, Tatsuya Shioda, Ayaka Tokumura, Satoshi Minami, Megumi Tsuchiya, Hidesato Ogawa, Maho Hamasaki, Li Yu, Yoshitaka Isaka, Tamotsu Yoshimori, and Shuhei Nakamura
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. In particular, reviewer #1 indicated that there were some inconsistencies in your writing. I hope that you will be address reviewer 1's concerns with further editing, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging.If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors provide a new mode for TFEB regulation

Comments for the author

The data presentation is extremely confusing. For example, Page 4, line 106 the authors stated that THOC4 knockdown significantly decreased autophagic activity. The authors showed that THOC4 KD, however, increases autophagy activity by repressing mTOR activity in the result section. They showed that THOC4 KD causes a reduction in TFEB protein level by destabilizing TFEB mRNAs. Depletion of THOC4 or TFEB does not affect autophagic flux by a 2 h starvation but causes a reduction in cells starved for 12 h. TFEB is involved in lysosomal biogenesis and function, which appears to be partially affected by THOC4 or TFEB KD. Long starvation causes drastic autophagy induction that might consume all lysosomes undergoing fusion with autophagosomes.
How does THOC4 regulate TFEB mRNA stability? The authors showed that the role of THOC4 in regulating TFEB mRNA is evolutionarily conserved in C. elegans. Do TFEB and HLH30 show sequence similarity at the mRNA level?

Reviewer 2

Advance summary and potential significance to field

The authors responded to all of my concerns I raised for the original manuscript in a satisfactory manner.

Comments for the author

The authors responded to all of my concerns I raised for the original manuscript in a satisfactory manner.

Reviewer 3

Advance summary and potential significance to field

The authors described how THOC4 interacts with TFEB mRNA to regulate the level of TFEB protein by stabilizing its mRNA.

Comments for the author

The authors answered issues raised by the reviewer. The reviewer believes that the paper met the requirement to publish in JCS.

Second revision

Author response to reviewers' comments

Reviewer's comments
Reviewer 1 Comments for the Author:
The data presentation is extremely confusing. For example, Page 4, line 106, the authors stated that THOC4 knockdown significantly decreased autophagic activity. The authors showed that THOC4 KD, however, increases autophagy activity by repressing mTOR activity in the result section.

Response
First of all, we thank the reviewer for investing tremendous efforts and times to review our paper and providing very valuable comments. We are very sorry for confusing you, but we were intended

to claim THOC4 has dual roles for autophagy depending on the length of starvation as stated in line 267-269.
While THOC4 knockdown increases the autophagic activity in the nutrient-rich condition through mTOR dependent, TFEB independent mechanism (Fig.S1 and S2), THOC4 knockdown decreases the autophagy flux during 12h prolonged starvation (Fig.4 C,D). To avoid such confusion, in the revised version of our manuscript, we clearly stated the condition in which THOC4 regulates autophagy.
The sentence in the introduction was changed to the following:
THOC4 knockdown significantly decreased autophagic activity and lipid breakdown during long-term starvation condition.

In addition, we inserted the following sentence to highlight the dual function of THOC4 at the end of introduction:
In addition, we found that THOC4 knockdown increases autophagy activity only during the nutrient rich condition independently of TFEB function suggesting that the dual roles of THOC4.

In addition, in the discussion section, we move some sentences discussing about possible TFEB independent function. The changed sentence is highlighted in yellow as well.

We hope these changes are fine. Thank you.

They showed that THOC4 KD causes a reduction in TFEB protein level by destabilizing TFEB mRNAs. Depletion of THOC4 or TFEB does not affect autophagic flux by a 2 h starvation, but causes a reduction in cells starved for 12 h. TFEB is involved in lysosomal biogenesis and function, which appears to be partially affected by THOC4 or TFEB KD. Long starvation causes drastic autophagy induction that might consume all lysosomes undergoing fusion with autophagosomes.

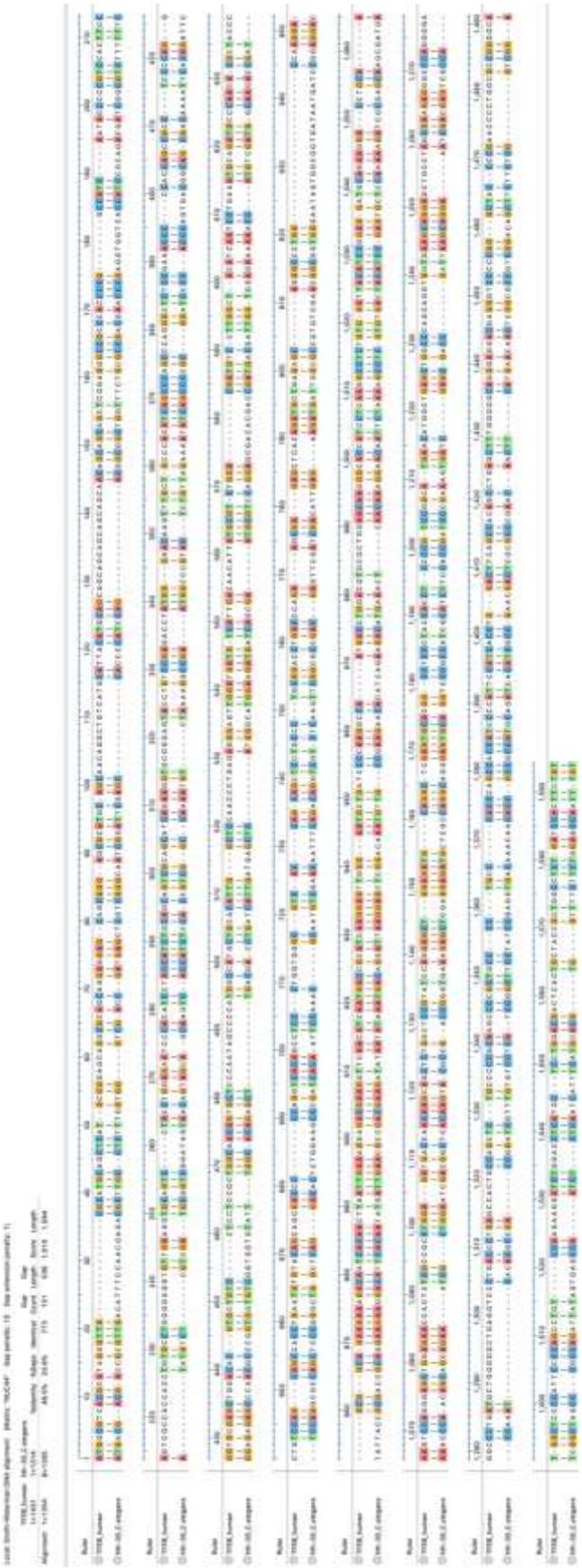
Response
Thank you very much for pointing that out and providing the possible mechanistic insight. We also would like to understand why THOC4 knockdown decreases autophagic activity only during long-term starvation. Based on the previous report (Yu et al., *Nature*, 2010), when starvation is prolonged, autophagic activity is reduced because of the reactivation of mTOR. This mTOR reactivation is essential for the lysosome reformation to keep the number of functional lysosomes. So far, at least, we confirmed that the mTOR was reactivated under the THOC4 or TFEB depleted conditions to the same extent as control (Fig.S3 E,F), suggesting that lysosomal reformation could be occurred. Preliminary results suggest that the number of LAMP1 positive or Lysotracker positive puncta were not significantly changed during long-term starvation in THOC4 knockdown compared to control. In addition, we found that the TFEB independent upregulation of several autophagy genes during the nutrient conditions in THOC4 knockdown was dampened in long-term starvation (Fig.S4), suggesting that autophagy induction by THOC4 is not present anymore at this condition. However we don't know if this is truly dependent on lack of TFEB function and would like to address the more detailed mechanism why THOC4 knockdown represses autophagic activity during long-term starvation in our future study. As you suggested, it is also plausible that the impairment of TFEB dependent lysosomal biogenesis ultimately affects autophagy activity. Thus, we added this points in the discussion section. The following is the inserted sentence:
Alternatively, reduced autophagic activity observed in THOC4 knockdown cells during the prolonged starvation might be due to lack of functional lysosomes. Sustained starvation together with lack of lysosomal biogenesis by TFEB depletion could consume all the functional lysosomes to be fused with autophagosomes.

Thank you!

How does THOC4 regulate TFEB mRNA stability? The authors showed that the role of THOC4 in regulating TFEB mRNA is evolutionarily conserved in C. elegans. Do TFEB and HLH30 show sequence similarity at the mRNA level?

Response
Thank you very much for your suggestion. HLH-30 has been previously shown to be a homolog of TFEB (Lapierre et al., Nat Commun, 2013). As you suggested, we compared the cDNA sequence of human *TFEB* to that of *C.elegans hlh-30*. The sequence similarity is about 48.5 % (pair wise

alignment, Smith-Waterman, DNAstar). The similar sequence could be the target for THOC4 (aly). The sequence alignment will be shown below and the consensus sequences were highlighted.



So far we revealed that exogenously expressed FLAG-TFEB and stably expressed mNG-TFEB, which do not have the original UTRs, were also dramatically decreased by THOC4 knockdown (Fig.2 B,C). These results suggest that the binding region could be in the coding region. Furthermore, THOC4 binds to mRNAs of other transcription factors to a similar extent (Fig.3 A,B) but the protein level (Fig.1 A,B) or mRNA stability of especially MITF was not largely affected by THOC4 knockdown (Fig.3 C,D). Since these differences in stability are thought to be due to differences in coding region sequences, we created multiple mutants that deletion in locations other than the common main domain, and examine whether they are affected by THOC4 KD. However, THOC4 KD reduced expression in all mutants tested (Fig. shown below). Higher-order structures of TFEB mRNA instead of the specific region might be essential to be recognized by THOC4, but we would like to address this point and how THOC4/aly regulates TFEB/HLH-30 mRNA stability in great detail in our upcoming studies.

Note: We have removed unpublished data provided for the referees in confidence.

Reviewer 2 Comments for the Author:
The authors responded to all of my concerns I raised for the original manuscript in a satisfactory manner.

Response
Thank you very much indeed for taking time to review our paper.

Reviewer 3 Comments for the Author:
The authors answered issues raised by the reviewer. The reviewer believes that the paper met the requirement to publish in JCS.

Response
Thank you very much indeed for taking time to review our paper.

Third decision letter

MS ID#: JOCES/2020/248203

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.