THE EMBO JOURNAL

Manuscript EMBO-2017-96699

STUB1 Regulates TFEB-Induced Autophagy-Lysosome Pathway

Youbao Sha, Lang Rao, Carmine Settembre, Andrea Ballabio & N. Tony Eissa

Corresponding author: N. Tony Eissa, Baylor College of Medicine

Review timeline:

Submission date:	08 February 2017
Editorial Decision:	28 February 2017
Revision received:	29 May 2017
Editorial Decision:	09 June 2017
Revision received:	21 June 2017
Accepted:	27 June 2017

Editor: Andrea Leibfried

Transaction Report:

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

1st Editorial Decision 28 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you will see, the referees appreciate your work. However, they also think that your conclusions are currently not sufficiently supported by the data provided, and that additional insight into the proposed interplay between STUB1, TFEB, and physiological cues is needed. I won't list the individual concerns here, as both reports are very clear and constructive.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

REFEREE REPORTS

Referee #1:

Sha et al demonstrate that STUB1, a chaperone -dependent E3 ubiquitin ligase, modulates TFEB

activity. STUB1 preferentially binds to phosphorylated TFEB and targets it for degradation by the ubiquitin proteasome pathway. They also showed that STUB1 could modulate TFEB activity by analyzing the activity of the PGC1a promoter, induction of autophagy and mitochondrial biogenesis. Based on their findings, they propose an interesting model wherein starvation (or mTOR inhibition) promotes the degradation of phosphorylated TFEB via STUB1, allowing non-phosphorylated TFEB to translocate to nucleus and exert its transcriptional function. In the absence of STUB1, phosphorylated TFEB could heterodimerize with non-phosphorylated TFEB, thus reducing its activity.
Overall, the data are supportive of the model. The studies provide a novel mechanism of TFEB regulation via control of its turnover.

Specific comments:
1. In Fig. 4, the presence of STUB1 enhanced levels of TFEB in the nucleus during starvation. The authors should include analysis of phosphorylation (using their phosphoantibody) of TFEB to demonstrate that the enhanced levels in the nuclear fractions are indeed dephosphorylated and that the control conditions (nutrient-replete) contain phosphorylated TFEB.

2. Analysis of phosphorylation of TFEB should also be included in Figures 5B and C to support that starvation increases the pool of dephosphorylated TFEB.

3. The authors should also demonstrate that nonphosphorylatable TFEB (Ala-TFEB) have increased translocation to the nucleus and activity towards PGC1a promoter (Figure 4).

4. The paper would be strengthened by analysis of possible target sites on TFEB for STUB-mediated ubiquitination.

Referee #2:

Sha et al. identify the autophagy-regulating transcription factor TFEB as a target of the chaperone-dependent ubiquitin ligase STUB1. The authors provide evidence that STUB1 preferentially targets phosphorylated TFEB for degradation by the proteasome. STUB1-dependent degradation of phosphorylated TFEB is shown to promote the activity of TFEB, thus facilitating the function of the transcription factor in the regulation of autophagy and mitochondrial biogenesis. The experiments are well controlled and largely support the drawn conclusions. However, the regulated interplay between STUB1 and TFEB under starvation conditions does not appear to be sufficiently investigated and some findings actually seem to contradict the main conclusion of the manuscript.

- Figure 5B: Although the basal level of TFEB is higher in the STUB1 depleted cells, the reduction of TFEB levels during starvation is similar to the one observed in control cells. It cannot be concluded that STUB1 degrades TFEB under starvation based on this experiment.

- Figure 5C: The finding that more TFEB is associated with STUB1 under starvation conditions or upon inhibition of mTOR is very confusing. Form other experiments the authors conclude that STUB1 preferentially interacts with phosphorylated, inactive TFEB. However, there should be less phosphorylated TFEB under starvation conditions or upon mTOR inhibition. So why is the interaction facilitated here? This seems to contradict the other conclusions of the authors and points to an additional regulatory mechanism that controls TFEB-STUB1 interaction in response to physiological cues. Additional experiments are needed in this regard. How does the phosphorylation status change under these conditions? Does STUB1 recognize unphosphorylated TFEB under starvation conditions?

- Figure 8: It is important to show that impaired mitochondrial biogenesis upon STUB1 depletion or ko can be rescued by expression of constitutively active TFEB. However, effects observed in HeLa cells are quite small. Why do the authors not use their MEFs with the more pronounced phenotype?

Manuscript EMBOJ-2017-96699
We appreciate the reviewers' comments and we provide point-by-point responses

Reviewer #1:
Reviewer: Sha et al demonstrate that STUB1, a chaperone -dependent E3 ubiquitin ligase, modulates TFEB activity. STUB1 preferentially binds to phosphorylated TFEB and targets it for degradation by the ubiquitin proteasome pathway. They also showed that STUB1 could modulate TFEB activity by analyzing the activity of the PGC1a promoter, induction of autophagy and mitochondrial biogenesis. Based on their findings, they propose an interesting model wherein starvation (or mTOR inhibition) promotes the degradation of phosphorylated TFEB via STUB1, allowing non-phosphorylated TFEB to translocate to nucleus and exert its transcriptional function. In the absence of STUB1, phosphorylated TFEB could heterodimerize with non-phosphorylated TFEB, thus reducing its activity. Overall, the data are supportive of the model. The studies provide a novel mechanism of TFEB regulation via control of its turnover.
Response: We agree with the reviewer.

Reviewer: Specific comments:
1. In Fig. 4, the presence of STUB1 enhanced levels of TFEB in the nucleus during starvation. The authors should include analysis of phosphorylation (using their phosphoantibody) of TFEB to demonstrate that the enhanced levels in the nuclear fractions are indeed dephosphorylated and that the control conditions (nutrient-replete) contain phosphorylated TFEB
Response: In the revised manuscript, we have included Appendix Figure S2B, which revealed that the increase in nuclear TFEB, in response to starvation, was non-phosphorylated TFEB.

Reviewer: 2. Analysis of phosphorylation of TFEB should also be included in Figures 5B and C to support that starvation increases the pool of dephosphorylated TFEB.
Response: In the revised manuscript, we have included this analysis in Figure 5B and C, which reveal that starvation increase the pool of dephosphorylated TFEB.

Reviewer: 3. The authors should also demonstrate that nonphosphorylatable TFEB (Ala-TFEB) have increased translocation to the nucleus and activity towards PGC1a promoter (Figure 4).
Response: In the revised manuscript, we have included this analysis in Appendix Figure S3, which revealed that nonphosphorylatable TFEB (Ala-TFEB) localized predominantly in the nucleus, and that it had increased activity towards PGC1a promoter.

Reviewer: 4. The paper would be strengthened by analysis of possible target sites on TFEB for STUB-mediated ubiquitination.
Response: We agree with the reviewer that pursuing this analysis is the next step. However, the scope of this type of detailed analysis would need to be included in a future independent study.

Reviewer #2
Reviewer: Sha et al. identify the autophagy-regulating transcription factor TFEB as a target of the chaperone-dependent ubiquitin ligase STUB1. The authors provide evidence that STUB1 preferentially targets phosphorylated TFEB for degradation by the proteasome. STUB1-dependent degradation of phosphorylated TFEB is shown to promote the activity of TFEB, thus facilitating the function of the transcription factor in the regulation of autophagy and mitochondrial biogenesis. The experiments are well controlled and largely support the drawn conclusions.
Response: We agree with the reviewer.

Reviewer: However, the regulated interplay between STUB1 and TFEB under starvation conditions does not appear to be sufficiently investigated and some findings actually seem to contradict the main conclusion of the manuscript.
- Figure 5B: Although the basal level of TFEB is higher in the STUB1 depleted cells, the reduction of TFEB levels during starvation is similar to the one observed in control cells. It cannot be concluded that STUB1 degrades TFEB under starvation based on this experiment.
Response: In the revised manuscript, we provide data, in Figure 5B, on the changes in both TFEB and phospho-TFEB in response to starvation in control cells and in STUB1-deficient cells. The data

revealed that the reduction of phosphor-TFEB during starvation was attenuated in STUB-1 deficient cells, consistent with a role of STUB-1 in phospho-TFEB mediated degradation.

Reviewer: - Figure 5C: The finding that more TFEB is associated with STUB1 under starvation conditions or upon inhibition of mTOR is very confusing. Form other experiments the authors conclude that STUB1 preferentially interacts with phosphorylated, inactive TFEB. However, there should be less phosphorylated TFEB under starvation conditions or upon mTOR inhibition. So why is the interaction facilitated here? This seems to contradict the other conclusions of the authors and points to an additional regulatory mechanism that controls TFEB-STUB1 interaction in response to physiological cues. Additional experiments are needed in this regard. How does the phosphorylation status change under these conditions? Does STUB1 recognize non-phosphorylated TFEB under starvation conditions?
Response: We appreciate the reviewer's comments and would like to clarify this issue. TFEB is phosphorylated and sequestered in cytoplasm in nutrient replete cells. Upon starvation or mTOR inhibition, there is a need for non-phosphorylated (active) TFEB to translocate to the nucleus. Our data suggested it occurred by targeting phosphorylated TFEB for degradation through increased interaction between STUB1 and phosphorylated TFEB. The remaining non-phosphorylated TFEB can freely associate into homodimer and translocate to the nucleus and increased its own transcription to produce additional non-phosphorylated TFEB. Our data show that STUB1 mediated degradation of phosphorylated TFEB was indeed accompanied by the activation of TFEB upon starvation or mTOR inhibition. Importantly, in STUB1 deficient cells, activation of TFEB by starvation was attenuated (Figure 4D).

Reviewer: - Figure 8: It is important to show that impaired mitochondrial biogenesis upon STUB1 depletion or ko can be rescued by expression of constitutively active TFEB. However, effects observed in HeLa cells are quite small. Why do the authors not use their MEFs with the more pronounced phenotype?
Response: In the revised manuscript, we have included this analysis in Figure EV5. The data show that reduced autophagy and impaired mitochondrial biogenesis in STUB1 deficient MEFs could be rescued by expression of constitutively active TFEB.

2nd Editorial Decision 09 June 2017

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are now both in favor of publication.

REFEREE REPORTS

Referee #1:

In this revision, the authors provide additional supporting evidence that phosphorylated TFEB is targeted by STUB and that dephosphorylated TFEB undergoes nuclear localization and has enhanced activity. While the target sites in TFEB are not known yet, the paper provide strong evidence on the role of STUB in TFEB regulation. The mechanism is quite interesting and the data are well presented.

Referee #2:

My concerns have been addressed and I recommend publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Eissa, N. Tony
The EMBO Journal
2017-96699R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were done at least three indepenent times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used cells and tissues from mice. All experiments were done at least three independent times.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Contorl and treated samples were tested side by side in all experiments.
For animal studies, include a statement about randomization even if no randomization was used.	The study did not involve randomization.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	The study did not involve blinding.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We used antibodies that were referenced in prior studies. These references are in the manuscript.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The sources of cell lines are in the methods section of the manuscript.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The information is provided in the methods section of the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments on animals were in compliance with ethical regulations and were approved by Instiutional Review Committee for Care of Animals.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance is confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No