EMBO reports

# The deubiquitinase OTUD1 enhances iron transport and potentiates host antitumor immunity

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## Review Timeline:


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| Submission Date: | 8th Jul 20 |
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

## 1st Editorial Decision 30th Jul 2020

Dear Dr. You,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Very importantly, as indicated also by the referees, please have your manuscript carefully proofread by a native speaker before re-submission.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these

should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

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Referee #1:

In the present study Song and colleagues identified OTUD1 as a deubiquitinase acting on IREB2

(Iron-responsive element-binding protein 2). They propose that the OTUD1-IREB2-transferrin receptor 1 (TfR1) axis acts as a tumour suppressor. Mechanistically the authors propose that by increasing intratumoral iron content, OTUD1 can induce ferroptosis, which, according to the authors, has immunogenic features. The work is interesting, and the data is overall convincing. In some instances, the work is very speculative, and this is not properly acknowledged. Therefore I have a series of remarks the authors might wish to address in order to improve the soundness of their work.

Major points:
1- Currently, the authors cannot exclude that OTUD1 could also act on TfR - this should be excluded. It would be helpful to generate OTUD1/IREB2 deficient cells and use their reconstituted system to study TfR levels - this should provide a stronger mechanistic link.
2- Additionally, the contribution of ferroptosis to the tumour suppressing function of OTUD1 is not convincing. Per definition, ferroptosis would require lipid peroxidation to take place, and at present, it's not clear if lipid peroxidation is contributing to the effect observed. I see two potential ways the authors could try to address this:
- Does liproxistatin-1, an in vivo active suppressor of ferroptosis, rescue the tumour growth of OTUD1 overexpression?
- Alternatively, can the removal of vitamin E from the diet increase the tumour suppressing function of OTUD1 overexpression?
These approaches should support a functional link between OTUD1 activity and increased lipid peroxidation in vivo. Without this, the contribution of ferroptosis is very speculative and should at least be toned down from the discussion.

Minor points:
-Citations
- The authors cite Sousa et al. to refer to the Fenton reaction, this is inadequate, and the authors should cite works discussing the role of iron and H2O2 specifically.
- When citing ferroptosis, the authors use a review by Cao&Dixon and Xie et al., they are both not up to date, and there are more recent one. Also, why the authors cite the work of Badgley is not clear.
Figure 1 - it's not clear how the authors define the signature "normal" and "dysfunctional iron homoeostasis".
Figure 1E - The authors indicate in the text that the pulldown was made from the colon - this is not clear, was it a colon cancer cell line? Do the authors use a mouse expressing Flag-IREB2? In the materials and methods, the authors only describe pulldowns made in HEK293T cells.
The pulldown experiments the authors should also add a loading control, such as beta-actin, for their Input samples and not only the bait.
Throughout the figures, the authors use the term "Untreated", which is incorrect - I would suggest switching to "Control".
In Figure 2C, 3C and 4A the authors should also blot for TfR1
The authors should also present the NOD-SCID mice data. Additionally, it's not clear from the text if they also used LCC cells for the xenografts.

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Referee #2:

The manuscript entitled "The deubiquitinase OTUD1 enhances iron transportation and potentiates host antitumor immunity" by Song et al, reports molecular mechanisms underlying regulation of IRE-

binding protein IREB2 by deubiquitinase OTUD1 and the relevance of the pathway in colorectal cancer development. In this piece of work authors make following central claims:
1. OTUD1 is identified as an interactor of IREB2 in cells and is shown to reduce its degradation and promote iron-regulatory effects such as cellular iron uptake by enhanced expression of TFRC.
2. Downregulation of OTUD1 expression is correlated with poor prognosis in context of colorectal cancer.
3. OTUD1 expression and the downstream IREB2-TFRC axis is critical for reinforcement of host anti-tumor immunity.

Although the clinical analyses included in the study are largely satisfactory, I have some reservation about the molecular connection between IREB2 and OTUD1 as outlined below.

Part 1: It is critical to show the role of FBXL5 in the OTUD1-IREB2 axis. FBXL5 is a well-documented E3-ubiquitin ligase which earmarks IREB2 for proteasomal degradation in response to alteration in cellular iron levels (Vashisht et al, 2009 and Salahudeen et al, 2009). Briefly, under high iron conditions, FBXL5, an iron sensing protein, binds iron and is stabilized. In this stable form FBXL5 recruits IREB2 to Skp1-Cul1-FBXL5 E3 ubiquitin ligase complex, as a consequence of which IREB2 is poly-ubiquitinated for subsequent degradation. On the other hand, under low iron conditions, FBXL5 is destabilized and itself undergoes proteasomal degradation leading to accumulation of IREB2. Since FBXL5 is known to be an important regulator of IREB2 under iron regulatory conditions, the present study in its current form is not complete without examining involvement of FBXL5 in IREB2-OTUD1 axis. The concerns in this relation are enlisted below.
Fig. 1 - Meta-analyses of GEO and TCGA databases convincingly identify the correlation between dysregulation of genes of iron metabolism and poor prognosis in colorectal cancer patients. However, it is not clear why OTUD1 was selected as the most important target in the IREB2 interactome in colon.
First of all, mass-spectrometric specifications used in the analyses are missing from results, figure legends and the methods sections. Second, it is not clear what conditions were used for obtaining and extracting lysates from mouse colon tissues and if the tissues were normal or cancerous. Third, and most importantly, as shown in Fig.1D, although the observation that IREB2 protein levels are downregulated in colorectal cancer tissues, in itself is convincing, however it begs the question of status of FBXL5 under same conditions. Did authors try to investigate role of FBXL5 in IREB2 regulation in context of colorectal cancer cell lines or tissues? It is especially important to investigate the role of FBXL5 in the IREB2-OTUD1 equation since in the present study this axis has been shown to be critical in context of iron-regulatory conditions. Without this validation, I'm skeptical of the independent relevance of the relationship between IREB2 and OTUD1 in the context of iron. Hence the authors should examine the impact of FBXL5 on IREB2-OTUD1 axis in, a) colorectal cancer versus normal tissues, b) under cellular changes in iron levels in cells of multiple lineage, and c) reciprocal effect of OTUD1 overexpression or depletion on FBXL5.
Fig.1E- For MS analysis, IREB2 is enriched separately from cell lines overexpressing it followed by incubation with lysates from colon tissues. This precludes any post-translational events that may be needed for regulation of IREB2 in colon tissues such as phosphorylation etc. which may be needed before the action of OTUD1. How have the authors taken this possibility under consideration?
Fig. EV2C- The authors show that treating cells with iron chelators such as DFO strengthen the interaction between IREB2 and OTUD1. Does the interaction between IREB2 and OTUD1 increase at the expense of reduction in the level of interaction between IREB2 and FBXL5? Under high iron conditions, FBXL5 is stabilized and causes degradation of IREB2. An important query that arises in context of the present study is what is the fate of IREB2-OTUD1 interaction under high iron conditions. Importantly, have the authors addressed the possibility of direct impact of FBXL5 on

OTUD1 under these conditions?
Fig EV2C- The stabilization of IREB2 may be due to loss of interaction between FBXL5 and IREB2 and decreased poly-ubiquitination of IREB2 under low iron conditions, since FBXL5 has compromised stability under these conditions. This aspect needs to be checked before concluding that the increased stability of IREB2 under low iron conditions is due to increased interaction between IREB2 and OTUD1. The contribution of FBXL5-mediated ubiquitination and OTUD1-mediated de-ubiquitination on IREB2 stability needs to be ascertained to remark upon the role of OTUD1 in regulating the stability of IREB2. One way to go about this question would be to study the interaction between IREB2 and OTUD1 and stabilizing effect of OTUD1 on IREB2 in cells depleted of FBXL5 under high and low iron conditions.
Fig. 1F- It is not clear how ubiquitination of IREB2 was induced to check the impact of OTUD1.
In Fig. EV2D, why is IREB2 so intensely ubiquitinated in cells transfected with K0-Ub? All lysine residues in Ub(K0) are substituted with arginine residues, thereby Ub(K0) can only support mono-ubiquitination, but not polyubiquitination. Can authors address this anomaly?
Fig.EV2E- Can the authors show on the blot the molecular weight of Flag-IREB2? The blots seem to be lacking high molecular weight polyubiquitinated forms of Flag-IREB2 which are expected in a cell-based polyubiquitination assay. The results are less than convincing in the absence of a clear smear-like pattern typical of polyubiquitinated forms of a substrate protein.
Fig.1G- In the protein half-life experiment, OTUD1 levels are severely reduced by 12 hours and are almost negligible by 24 hours. However, IREB2 levels are quite stable at 12 and 24 hours. How do the authors explain this discrepancy? Moreover, it will be interesting to study how OTUD1 protein and mRNA levels are affected by low and high iron treatments.
Fig. 1I- Hemin and AFC treatments appear to affect TFRC expression in OTUD-/- cells to a very small extent, the fold difference is less than 1.5 folds. There is no significant additional impact of OTUD-/- condition on TFRC expression beyond AFC/Hemin treatments.
Altogether, the results shown in Fig. 1 show that OTUD1 may have some positive effect on stability of IREB2, however fail to show convincingly the significance of this interaction on IREB2 stability under iron-regulatory conditions in comparison to the well-established IREB2-FBXL5 relationship.

Part 2- The authors' claim that OTUD1 mRNA expression is down-regulated in colorectal cancer tissues is well-supported by the data shown in Fig. 2. Did the authors compare and correlate mRNA expression of TFRC with that of FBXL5?
The Figure 3 data is executed with a rigorous panel of biochemical assays and animal experiments and I greatly applaud the authors' efforts in establishing the positive impact of OTUD1 on maintenance of iron transport.

Part 3- the data presented in Fig. 4C-E convincingly establish that OTUD1 expression in a tumor background suppresses tumor growth and increases the survival times of animals transplanted with OTUD1-expressing cells. Additionally, it is clear from Fig. 4F, G and J that OTUD1 overexpressing tumors attract more cytotoxic T-cells to the tumor micro-environment when compared to the control tumors, alluding to involvement of OTUD1 in promoting host T-cell response against cancer.
Fig.5A shows mass-spectrometry based evaluation of danger-associated molecular patterns expressed in the tumor interstitial fluid. No details of the mass-spectrometric analysis have not been provided in the methods section. HSP70 and HSP90 western blots (Fig. 5B) are less than convincing since the blots are oversaturated.
The data in Fig. 5G-L is supportive of the authors' claim that OTUD1 is involved in ferroptosis and its overexpression in tumors sensitizes cells to ferroptosis-mediated death induced by activators such as RSL3 and erastin.
In the end the authors tie in together the tumor volume analysis, lymphocyte characterization and ROS/ATP measurements in wild-type or OTUD1-/-colitis-associated cancer model. It is clear from

data presented in Fig. 6B and C that OTUD1 expression is negatively correlated with tumor load in animals. Further, authors satisfactorily show that through Fig. 6E that OTUD1 expression in tumors is positively correlated with maintenance of TFRC expression. Comparisons of levels of intracellular ROS and ATP leakage in the tumor interstitial fluid between wild-type and OTUD1-/- animals argue for a role of OTUD1 in development of colon cancer.

Other critiques related to manuscript structure:
The general structure of the manuscript lacks coherence, lucidity and succinctness, which makes it difficult to comprehend the reasoning and relevance of the experiment performed and conclusions drawn from the text alone. The prevalence of easily avoidable typos makes it hard for the reader to appreciate the reason, logic and importance of experiments and observations reported in the manuscript. The manuscript requires thorough editing both in terms of the language as well as scientific accuracy of the conclusions drawn from the observations.
There are lots of typos throughout the text, some examples are mentioned below:
Section "OTUD1 acts as a deubiquitinase of IREB2'- "...supplementation of Deferoxamine (DFO), the iron chelation,.."
Abstract- "..In spite of the essential element for life,...."
Discussion- "...there was no spontaneous tumor developed in Otud1-/- mice within one year old."

The abstract is poorly constructed with insufficient background information provided to build a case for lack of current understanding of role of iron in cancer-related immune response and how the authors' work contributes to filling these gaps. Importantly, the authors have failed to cite most recent and comprehensive reviews relevant to their research interest such as 2018 Annual review of nutrition "Iron and Cancer" published from Torti lab.

References:
1. Vashisht AA, Zumbrennen KB, Huang X, et al. Control of iron homeostasis by an iron-regulated ubiquitin ligase. Science. 2009;326(5953):718-721.
2. Salahudeen AA, Thompson JW, Ruiz JC, et al. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. Science. 2009;326(5953):722-726. doi:10.1126/science.1176326
3. Suzy V. Torti, David H. Manz, Bibbin T. Paul, et al. Iron and Cancer. Annual Review of Nutrition. 2018 38:1, 97-125.

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Referee #3:

This manuscript reports: (1) OTUD1 deubiquitinates and stabilizes IREB2, promoting TFRC-mediated iron transportation. (2) OTUD1 increases cellular iron uptake that triggers immunogenic cell death, leading to suppression of tumor growth. (3) Loss of OTUD1 impedes tumor-reactive T cell accumulation and promotes colon cancer progression. These findings are very interesting and the authors provided data to support their conclusions. The manuscript will be suitable for publication in EMBO reports if the authors can provide data to address the following concerns and strengthen their conclusions:

1. In vitro deubiquitination assays, with wild-type and catalytically inactive (C320S) OTUD1, should be used to demonstrate that OTUD1 can deubiquitinate IREB2 directly.
2. Figure 1F (in vivo deubiquitination assays): how to confirm that the Ub signal is indeed from IREB2, but not from IREB2-interacting proteins?

3. Is polyubiquitination of Ireb2 upregulated in Otud1-knockout mouse tissues?
4. Figure 3: rescue experiments and/or gain-of-function experiments, with the C320S mutant as the negative control, should be presented to demonstrate that OTUD1 promotes cellular iron uptake. Also, the authors should demonstrate that IREB2 mediates the role of OTUD1 in iron uptake.
5. Figures 4 & 5: the C320S mutant should be included as a negative control in these experiments. Can IREB2 knockdown reverse the effects of OTUD1 overexpression?
6. In those in vivo tumor growth experiments, the levels of ferroptosis, iron, lipid ROS should be examined and compared between different groups. This will be needed to prove that OTUD1 drives intracellular iron accumulation and promotes oxidative damage, which in turn augments ferroptosis.
7. Statistical analysis is lacking in Figure 2E.
8. The English of this manuscript needs editing.

We would like to thank the reviewers for their critical comments and insightful suggestions concerning our manuscript entitled "The deubiquitinase OTUD1 enhances iron transportation and potentiates host antitumor immunity" (Manuscript ID: EMBOR-2020-51162V1) that we submitted to *EMBO reports*. We have now made a thorough revision of the paper based on new data. The following is a point-by-point response to the reviewers' comments and questions.

Point-to-point response to the reviewers' comments is as followed:

We would first like to express our sincerely thanks to the reviewers for the positive comments and affirmation of our study. We found the reviewers' comments to be very helpful and of value for improving the quality of our data and manuscript. Accordingly, we have addressed each of the comments as follows.

Reviewers' comments:

*Referee #1:*

*In the present study Song and colleagues identified OTUD1 as a deubiquitinase acting on IREB2 (Iron-responsive element-binding protein 2). They propose that the OTUD1-IREB2-transferrin receptor 1 (TfR1) axis acts as a tumour suppressor. Mechanistically the authors propose that by increasing intratumoral iron content, OTUD1 can induce ferroptosis, which, according to the authors, has immunogenic features. The work is interesting, and the data is overall convincing. In some instances, the work is very speculative, and this is not properly acknowledged. Therefore I have a series of remarks the authors might wish to address in order to improve the soundness of their work.*

*Major points:*

*1- Currently, the authors cannot exclude that OTUD1 could also act on TfR - this should be excluded. It would be helpful to generate OTUD1/IREB2 deficient cells and use their reconstituted system to study TfR levels - this should provide a stronger mechanistic link.*

[**Response**] According to these suggestions, we used CRISPR-Cas9 to delete the *Ireb2* in CT26 cells to test whether the stimulatory effect of OTUD1 on TFRC expression is in an IREB2-dependent manner. Both western blot and flow cytometry assays revealed that overexpression of OTUD1 promoted TFRC expression in wild-type (WT) CT26 cells rather than *Ireb2*$^{-/-}$ cells (**Figs EV2G and H, Appendix Fig S2C**). Consistently, compared with WT cells, the presence of OTUD1 exerts little effects on iron absorption in *Ireb2*$^{-/-}$ cells treated with ammonium ferric citrate (AFC) (**Fig EV3A**). Collectively, our data demonstrate that IREB2 is required for the modulation of TFRC by OTUD1.

*2- Additionally, the contribution of ferroptosis to the tumour suppressing function of OTUD1 is not convincing. Per definition, ferroptosis would require lipid peroxidation to*

*take place, and at present, it's not clear if lipid peroxidation is contributing to the effect observed. I see two potential ways the authors could try to address this:*
*- Does liproxistatin-1, an in vivo active suppressor of ferroptosis, rescue the tumour growth of OTUD1 overexpression?*
*- Alternatively, can the removal of vitamin E from the diet increase the tumour suppressing function of OTUD1 overexpression?*
*These approaches should support a functional link between OTUD1 activity and increased lipid peroxidation in vivo. Without this, the contribution of ferroptosis is very speculative and should at least be toned down from the discussion.*

[**Response**] We are grateful for the reviewer's suggestions. In order to ascertain that ferroptosis mainly contributes to the tumor-suppressive function of OTUD1, we used vitamin E (VE), radical scavenger, to suppress ferroptosis commitment. Considering only a portion of VE dosage can reach the tumor by oral administration and its water insoluble characteristic, we exploited a molecular-matched strategy to prepare VE-loading TPGS nanoparticles (NP-VE) with extremely high drug loading levels (up to 10 mg/ml). The nanoparticles have particle sizes of 144.37±0.39 nm and zeta potentials of -29.43±0.46 mV determined by dynamic light scattering (DLS) methods (**Figs EV4D and E**). *In vitro* assay showed that NP-VE treatment curtailed the RSL3-induced ferroptosis in OTUD1-expressing cells (**Fig EV4F**).

We next intratumorally injected the NP-VE into mice bearing mock-expressing or OTUD1-expressing tumors (**Fig 5H**). As shown in **Figure 5I**, in contrast to the little effects on tumor growth in mice bearing mock-expressing tumor, NP-VE treatment blocked the inhibitory effects of OTUD1 on tumor growth. Moreover, flow cytometry analysis revealed that NP-VE treatment remarkably reduced the intracellular level of ROS in both mock and OTUD1-expressing tumors (**Fig 5J and Appendix Fig S6C**). Notably, supplementation of NP-VE also reduced the ratio of tumor-infiltrated $CD8^+$ T cell (**Fig 5K and Appendix Fig S6D**). Our data thus demonstrate that the stimulatory role of OTUD1 in ferroptosis drives host antitumor immunity and suppresses tumor development.

*Minor points:*
*-Citations*
*- The authors cite Sousa et al. to refer to the Fenton reaction, this is inadequate, and the authors should cite works discussing the role of iron and H2O2 specifically.*

[**Response**] As suggested, we have discussed the role of iron and ROS in ferroptosis in our revised manuscript.

*- When citing ferroptosis, the authors use a review by Cao&Dixon and Xie et al., they are both not up to date, and there are more recent one. Also, why the authors cite the work of Badgley is not clear.*

[**Response**] We agree that the papers we cited are not up to date. We have replaced the

"Cao & Dixon, 2016; Xie *et al*, 2016" with "Friedmann *et al*, 2019; Hassannia *et al*, 2019; Suzy *et al*, 2018". Besides, we have discussed the work of Badgley in our revised manuscript as the reviewer suggested.

*Figure 1 - it's not clear how the authors define the signature "normal" and "dysfunctional iron homoeostasis".*

[**Response**] As suggested, we have defined the signature "normal" and "dysfunctional iron homoeostasis" in the figure legend in our revised manuscript.

*Figure 1E - The authors indicate in the text that the pulldown was made from the colon - this is not clear, was it a colon cancer cell line? Do the authors use a mouse expressing Flag-IREB2? In the materials and methods, the authors only describe pulldowns made in HEK293T cells.*

[**Response**] We regret any confusion caused by our inaccurate statement. The pulldown in **Figure 1E** was made from the mice colon tissues rather than colon cancer cell line.
HEK293T cells were transfected with mock or IREB2-FLAG vector and the vehicle or IREB2 protein was purified by anti-FLAG M2 beads. Subsequently, these purified proteins were incubated with lysates from mice colon tissues, respectively. We have outlined this protocol in detail in the section of materials and methods in our revised manuscript.

*The pulldown experiments the authors should also add a loading control, such as beta-actin, for their Input samples and not only the bait.*

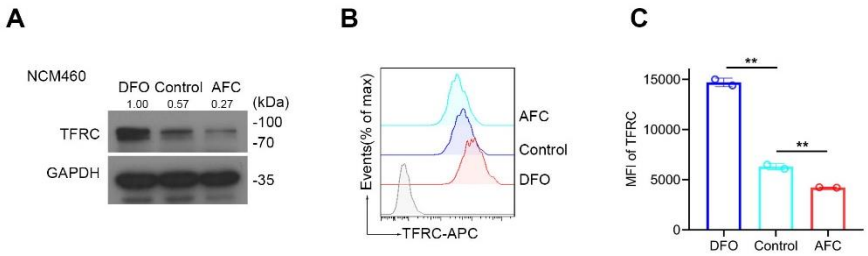
[**Response**] As suggested, we have added the loading control for all pulldown experiments in our revised manuscript.

*Throughout the figures, the authors use the term "Untreated", which is incorrect - I would suggest switching to "Control".*

[**Response**] As suggested, the term "Untreated" has been revised into "Control" in our revised manuscript.

*In Figure 2C, 3C and 4A the authors should also blot for TfR1*

[**Response**] As suggested, we employed western blot to assess the status of TFRC in cells treated with deferoxamine (DFO) or ammonium ferric citrate (AFC). Consistent to the results detected by flow cytometry, TFRC expression was stimulated by DFO in NCM460 cells, but attenuated in the treatment with AFC (**Response Fig 1**). Furthermore, as shown in **Figures 2C, 3C and 4F**, we also employed western blot assay to assess the status of TFRC in primary tumor tissues and murine colon tissues.




**Response Figure 1. Alteration of cellular iron level affects TFRC expression.**
(**A**) Western blot analysis of TFRC expression in NCM460 cells with treatment of DFO (100 μM) or AFC (50 μM).
(**B-C**) Flow cytometric analysis of TFRC expression in NCM460 cells in presence of DFO (100 μM) or AFC (50 μM). MFI, mean fluorescence intensity. Control uses isotype-matched control antibody (n = 2 cell cultures, mean ± s.e.m., $^{**}P < 0.01$).

*The authors should also present the NOD-SCID mice data. Additionally, it's not clear from the text if they also used LCC cells for the xenografts.*

[**Response**] As suggested, NOD-SCID mice data have been deposited in **Figures EV4B** and **C**. And we also pointed out in revised manuscript that we used mouse Lewis lung carcinoma cell line (LLC) as well as mouse colon cancer cell line (CT26) to investigate the role of OTUD1 in tumorigenesis.

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*Referee #2:*

*The manuscript entitled "The deubiquitinase OTUD1 enhances iron transportation and potentiates host antitumor immunity" by Song et al, reports molecular mechanisms underlying regulation of IRE-binding protein IREB2 by deubiquitinase OTUD1 and the relevance of the pathway in colorectal cancer development. In this piece of work authors make following central claims:*
*1. OTUD1 is identified as an interactor of IREB2 in cells and is shown to reduce its degradation and promote iron-regulatory effects such as cellular iron uptake by enhanced expression of TFRC.*
*2. Downregulation of OTUD1 expression is correlated with poor prognosis in context of colorectal cancer.*
*3. OTUD1 expression and the downstream IREB2-TFRC axis is critical for reinforcement of host anti-tumor immunity.*

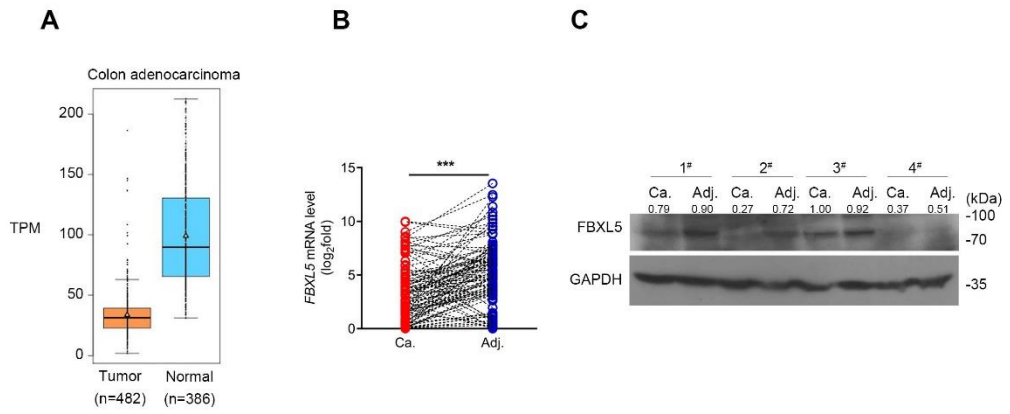
*Although the clinical analyses included in the study are largely satisfactory, I have some reservation about the molecular connection between IREB2 and OTUD1 as outlined below.*

*Part 1: It is critical to show the role of FBXL5 in the OTUD1-IREB2 axis. FBXL5 is a well-documented E3-ubiquitin ligase which earmarks IREB2 for proteasomal degradation in response to alteration in cellular iron levels (Vashisht et al, 2009 and Salahudeen et al, 2009). Briefly, under high iron conditions, FBXL5, an iron sensing protein, binds iron and is stabilized. In this stable form FBXL5 recruits IREB2 to Skp1-Cul1-FBXL5 E3 ubiquitin ligase complex, as a consequence of which IREB2 is poly-ubiquitinated for subsequent degradation. On the other hand, under low iron conditions, FBXL5 is destabilized and itself undergoes proteasomal degradation leading to accumulation of IREB2. Since FBXL5 is known to be an important regulator of IREB2 under iron regulatory conditions, the present study in its current form is not complete without examining involvement of FBXL5 in IREB2-OTUD1 axis. The concerns in this relation are enlisted below.*

*Fig. 1 - Meta-analyses of GEO and TCGA databases convincingly identify the correlation between dysregulation of genes of iron metabolism and poor prognosis in colorectal cancer patients. However, it is not clear why OTUD1 was selected as the most important target in the IREB2 interactome in colon.*

[**Response**] We thank the reviewer for this question. Our present results showed that the protein level of IREB2, rather than its mRNA level, was downregulated in primary colon cancer tissues as relative to their matched normal tissues (**Fig 1C and Figs EV1D** and **E**), which suggested that downregulation of IREB2 in cancer is largely attribute to the post-translational regulation. Furthermore, we used anti-IREB2 antibody to pulldown the endogenous IREB2 from primary colon cancer tissues or their matched normal colon tissues. Subsequently, we used anti-ubiquitin antibody to assess the ubiquitination modification of IREB2. As shown in **Figure 1D**, the ubiquitination of IREB2 was increased in cancers as compared with that in normal tissues.

As the reviewer mentioned above, the E3 ubiquitin ligase FBXL5 is known to target IREB2 for proteasome degradation. We thus interrogated the transcriptional data of *FBXL5* from TCGA database and found that the mRNA level of *FBXL5* was also downregulated in colon cancers (**Response Fig 2A**). To further confirm this result, we employed RT-qPCR and western blot assays to detect the mRNA and the protein level of FBXL5, respectively. As shown in **Response Figures 2B** and **C**, the FBXL5 was downregulated in cancerous tissues as relative to their matched normal tissues. In the light of the suppressive role of FBXL5 in regulation of IREB2, downregulation of FBXL5 may promote rather than decrease IREB2 expression in cancers. Our data thus indicate other post-translational mechanisms contribute to the downregulation of IREB2 in cancers. Through analysis of the IREB2 interactome in colon, we identified the deubiquitinase OTUD1 that can catalytically remove the ubiquitin from substrate proteins and thus selected OTUD1 as the most potential target in the IREB2 interactome.




**Response Figure 2. FBXL5 is downregulated in colon cancers.**
(**A**) The transcript of *FBXL5* was reduced in colon adenocarcinoma analyzed by TCGA database (Data was downloaded from http://gemini.cancer-pku.cn/).
(**B**) RT–qPCR analysis of *FBXL5* mRNA levels in colon tumors and matched adjacent normal tissues (n = 101 human samples, mean ± s.e.m., ***$P < 0.01$).
(**C**) Western blot analysis of FBXL5 expression in colon cancers and matched adjacent normal tissues.

*First of all, mass-spectrometric specifications used in the analyses are missing from results, figure legends and the methods sections.*

[**Response**] As suggested, we have added the mass-spectrometric specifications in the methods section in the revised manuscript.

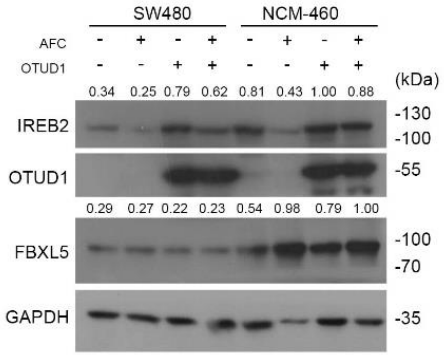
*Second, it is not clear what conditions were used for obtaining and extracting lysates from mouse colon tissues and if the tissues were normal or cancerous.*

[**Response**] As suggested, we have outlined this protocol in detail in the section of materials and methods as well as figure legends in our revised manuscript.

*Third, and most importantly, as shown in Fig.1D, although the observation that IREB2 protein levels are downregulated in colorectal cancer tissues, in itself is convincing, however it begs the question of status of FBXL5 under same conditions. Did authors try to investigate role of FBXL5 in IREB2 regulation in context of colorectal cancer cell lines or tissues?*

[**Response**] The following efforts were made to address this concern. As mentioned above, FBXL5 was downregulated in cancerous tissues as relative to their matched normal tissues, which is consistent with the result interrogated from TCGA database (**Response Fig 2**). Furthermore, we also compared the expression of FBXL5 between SW480 and NCM460, which are colorectal cancer cell line and human non-cancerous

colonic epithelial cell line, respectively. As shown in **Response Figure 3**, higher level of FBXL5 was detected in NCM460 than that in SW480 cell line. More importantly, in contrast to the stimulatory effects of ammonium ferric citrate (AFC) on FBXL5 expression in NCM460, AFC treatment elicited little effects on FBXL5 expression in SW480 cells (**Response Fig 3**). Our data thus indicate that FBXL5 is inactivated during colon cancer development. Besides, in the light of the critical role of FBXL5 in modulation of iron metabolism, we have reported the status of FBXL5 in colon cancers in the section of discussion.




**Response Figure 3. OTUD1 enhances IREB2 expression in a FBXL5-independent manner.**
SW480 or NCM460 cells transfected with the indicated vectors were treated with AFC (50 μM) or not. The expression of indicated proteins were measure by western blots and the band intensity was quantified by *Image J* software.

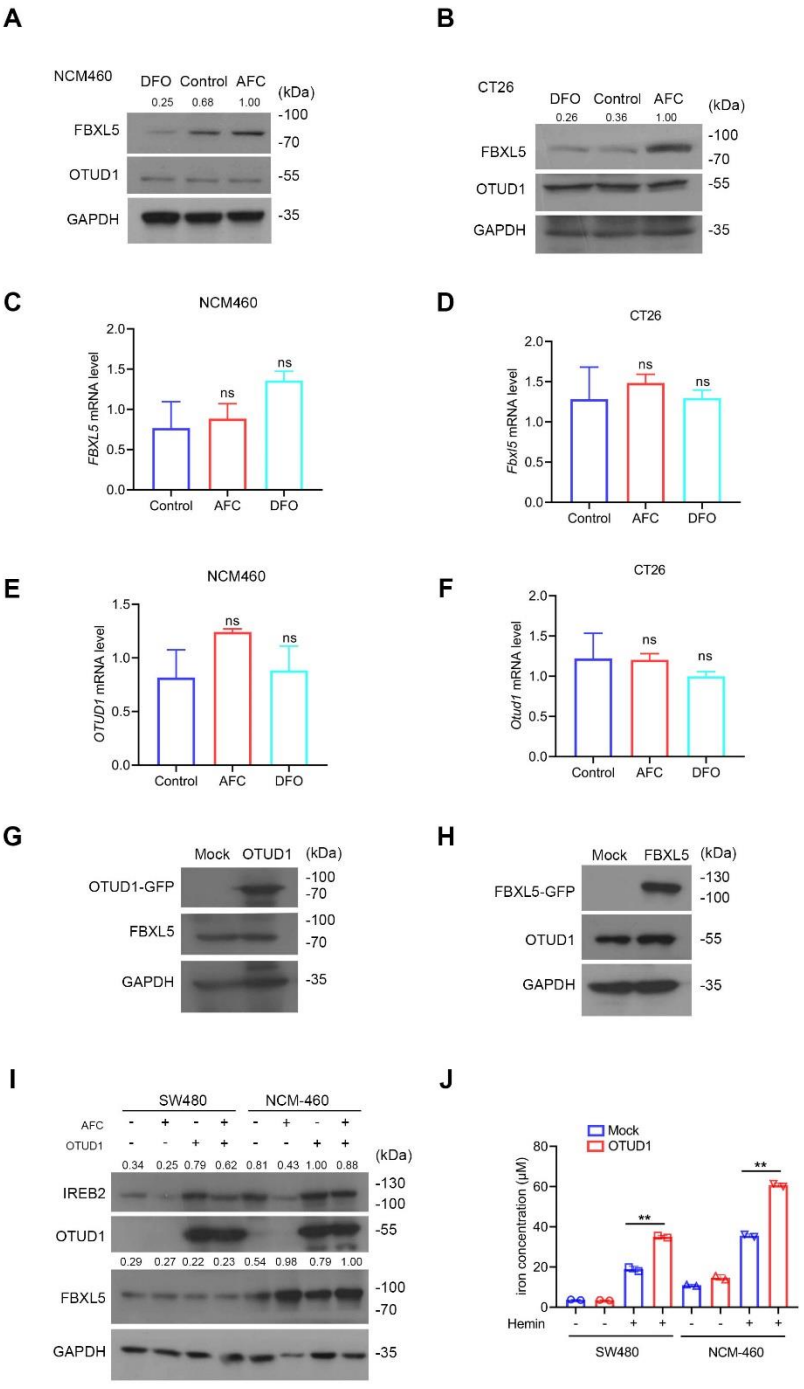
*It is especially important to investigate the role of FBXL5 in the IREB2-OTUD1 equation since in the present study this axis has been shown to be critical in context of iron-regulatory conditions. Without this validation, I'm skeptical of the independent relevance of the relationship between IREB2 and OTUD1 in the context of iron. Hence the authors should examine the impact of FBXL5 on IREB2-OTUD1 axis in, a) colorectal cancer versus normal tissues, b) under cellular changes in iron levels in cells of multiple lineage, and c) reciprocal effect of OTUD1 overexpression or depletion on FBXL5.*

[**Response**] The following efforts were made to address this concern. In order to measure the impact of FBXL5 on IREB2-OTUD1 axis, we firstly employed deferoxamine (DFO) or ammonium ferric citrate (AFC) to stimulate NCM460 and CT26 cells. As shown in **Response Figure 4A**, the expression of FBXL5 was increased under AFC treatment but reduced in the treatment of DFO in NCM460 cells. Despite the less sensitivity to DFO treatment, FBXL5 can be induced by AFC treatment in CT26 cells (**Response Fig 4B**). In accidence with previous reports, the protein level of FBXL5 rather than its mRNA level can be induced in context of iron-regulatory conditions (**Response Figs 4C and D**). Unlike the inducible role of FBXL5, both mRNA and protein level of OTUD1 were stable (**Response Figs 4A, B,**

**E and F**).

To test whether overexpression or depletion of OTUD1 can affect FBXL5 expression, we transfected OTUD1 or mock vector into NCM460 cell line and assessed the endogenous level of FBXL5. As shown in **Response Figure 4G**, enforced expression of OTUD1 elicited little effects on FBXL5 expression. Moreover, we also assesseed the status of FBXL5 in colon from *Otud1*$^{-/-}$ mice. As shown in **Figure 3C**, although loss of OTUD1 impaired IREB2 and TFRC expression, the FBXL5 expression in *Otud1*$^{-/-}$ mice was hardly affected as compared with wild-type mice. Moreover, FBXL5 expression is identical in CT26 tumors overexpressing mock, OTUD1 or OTUD1$^{C320S}$ (**Fig 4F**).

To test whether FBXL5 can regulate OTUD1 expression or function, we firstly transfected FBXL5 or mock vector into NCM460 cell line and detected the expression of OTUD1. As shown in **Response Figure 4H**, overexpression of FBXL5 hardly affected the endogenous expression of OTUD1 in NCM460 cells. Additionally, we investigated the function of OTUD1 in NCM460 and SW480, whose FBXL5 can be induced by AFC treatment or not, respectively. As shown in **Response Figure 4I**, in contrast to the downregulation of IREB2 by AFC treatment, enforced expression of OTUD1 increased the protein level of IREB2 in both FBXL5-active and inactive cell lines. In addition, FBXL5 has little effects on OTUD1 mediated iron transportation (**Response Fig 4J**). Our data thus demonstrate that the stabilizing effects of OTUD1 on IREB2 is in an FBXL5-independent manner.




**Response Figure 4. Alteration of cellular iron concentration hardly affects OTUD1 expression.**

(**A-B**) NCM460 or CT26 cells were treated with DFO (100 μM) or AFC (50 μM), respectively. 24hrs later, the protein levels of FBXL5 and OTUD1 were measured by western blot assay, separately.

(**C-D**) RT–qPCR analysis of the mRNA levels of *FBXL5* in NCM460 cells and CT26

cells treated with DFO (100 μM) or AFC (50 μM), respectively. (n = 2 cell cultures, mean ± s.e.m., ns > 0.05).
(**E-F**) RT–qPCR analysis of the mRNA levels of *OTUD1* in NCM460 cells and CT26 cells treated with DFO (100 μM) or AFC (50 μM), respectively. (n = 2 cell cultures, mean ± s.e.m., ns > 0.05).
(**G-H**) OTUD1 or FBXL5 vector was transfected into NCM460 cells. 24hrs later, the endogenous FBXL5 or OTUD1 was measured by western blot assay, separately.
(**I**) SW480 or NCM460 cells transfected with the indicated vectors were treated with AFC (50 μM) or not. The expression of indicated proteins were measure by western blot and the band intensity was quantified by *Image J* software.
(**J**) Intracellular iron concentration was measured in SW480 and NCM460 cells transfected with mock or OTUD1 vector in presence or absence of Hemin treatment (n = 2 cell cultures, mean ± s.e.m., $^{**}P < 0.01$).

*Fig.1E- For MS analysis, IREB2 is enriched separately from cell lines overexpressing it followed by incubation with lysates from colon tissues. This precludes any post-translational events that may be needed for regulation of IREB2 in colon tissues such as phosphorylation etc. which may be needed before the action of OTUD1. How have the authors taken this possibility under consideration?*

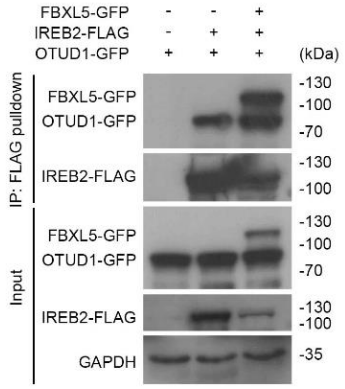
[**Response**] We are grateful for the reviewer's suggestions. Considering that the protein level of IREB2 rather than its mRNA level was downregulated in primary colon cancer tissues, we thus attributed the downregulation of IREB2 in cancer to the protein instability. It is well-known that protein degradation is largely mediated by the ubiquitin-proteasome system, we therefore used anti-IREB2 antibody to pulldown the endogenous IREB2 from primary colon cancer tissues or their matched normal colon tissues. Subsequently, we used anti-ubiquitin antibody to assess the ubiquitination of IREB2. As shown in **Figure 1D**, the ubiquitination of IREB2 was increased in cancers as compared with that in normal tissues, which suggested that instability of IREB2 is largely attribute to the ubiquitination modification. Theoretically, other post-translational events may be involved in the regulation of IREB2, we will continue to study other potential modifications of IREB2 in our future works.

*Fig. EV2C- The authors show that treating cells with iron chelators such as DFO strengthen the interaction between IREB2 and OTUD1. Does the interaction between IREB2 and OTUD1 increase at the expense of reduction in the level of interaction between IREB2 and FBXL5? Under high iron conditions, FBXL5 is stabilized and causes degradation of IREB2. An important query that arises in context of the present study is what is the fate of IREB2-OTUD1 interaction under high iron conditions. Importantly, have the authors addressed the possibility of direct impact of FBXL5 on OTUD1 under these conditions?*

[**Response**] We are grateful for the reviewer's suggestion. As mentioned above, enforced expression of FBXL5 hardly affected OTUD1 expression in NCM460. To

determine whether presence of FBXL5 can affect the interaction between OTUD1 and IREB2, we co-transfected OTUD1, FBXL5 and IREB2 into HEK293T cells. As shown in **Response Figure 5**, presence of FBXL5 hardly affected the association of IREB2 with OTUD1.

In addition to iron-binding activity, IREB2 is also an RNA-binding protein, which selectively binds to the mRNA regions containing iron-responsive elements (IRES). It is known that iron-binding impairs IREB2 RNA-binding activity, thereby inducing IRES-containing mRNA decay. In our present study, we found that DFO treatment remarkably strengthened the association of IREB2 with OTUD1 (**Fig EV2C**). Considering the stable expression of OTUD1 upon exposure to alteration of iron concentration, we speculated that iron-binding not only impaired the RNA-binding activity of IREB2 but also attenuated its association with OTUD1.

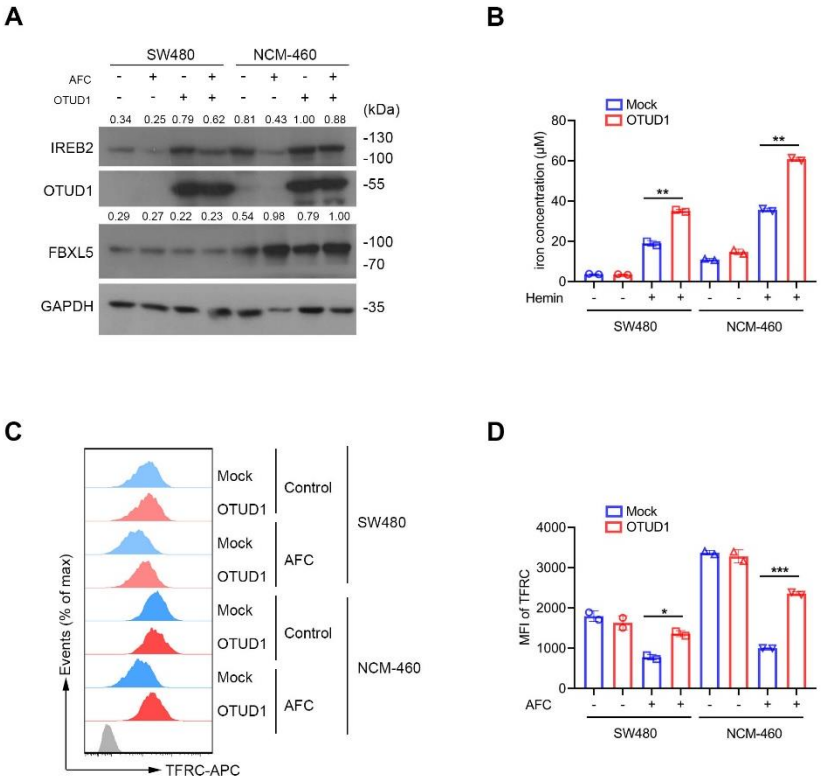



**Response Figure 5. FBXL5 exerts little effects on the relationship between OTUD1 and IREB2.**
HEK293T cells were co-transfected with indicated vectors. 24hrs later, cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblot with anti-GFP antibody.

*Fig EV2C- The stabilization of IREB2 may be due to loss of interaction between FBXL5 and IREB2 and decreased poly-ubiquitination of IREB2 under low iron conditions, since FBXL5 has compromised stability under these conditions. This aspect needs to be checked before concluding that the increased stability of IREB2 under low iron conditions is due to increased interaction between IREB2 and OTUD1. The contribution of FBXL5-mediated ubiquitination and OTUD1-mediated de-ubiquitination on IREB2 stability needs to be ascertained to remark upon the role of OTUD1 in regulating the stability of IREB2. One way to go about this question would be to study the interaction between IREB2 and OTUD1 and stabilizing effect of OTUD1 on IREB2 in cells depleted of FBXL5 under high and low iron conditions.*

[**Response**] We are grateful for the reviewer's suggestion. To determine whether FBXL5 is involved in the OTUD1-mediated regulation of IREB2, we used NCM460

and SW480, which are FBXL5-active and inactive cell line, respectively. As shown in **Response Figure 6A**, in contrast to the downregulation of IREB2 by AFC treatment, enforced expression of OTUD1 increased the protein level of IREB2 in both FBXL5-active and inactive cell lines. In addition, OTUD1 promotes TFRC level and iron transportation in both cell lines (**Response Figs 6B-D**). Our data thus demonstrate that the stabilizing effects of OTUD1 on IREB2 is in a FBXL5-independent manner.




**Response Figure 6. FBXL5 exerts little effects on OTUD1-IREB2-TFRC signaling.**

(**A**) SW480 or NCM460 cells transfected with the indicated vectors were treated or untreated with AFC (50 μM). The expression of indicated proteins were measure by western blots and the band intensity was quantified by *Image J* software.

(**B**) Intracellular iron concentration was measured in SW480 and NCM460 cells transfected with mock or OTUD1 vector in presence or absence of Hemin treatment (n = 2 cell cultures, mean ± s.e.m., $^{**}P < 0.01$).

(**C-D**) Flow cytometric analysis of TFRC expression in SW480 or NCM460 cells transfected with the indicated vectors were treated with AFC (50 μM) or not. MFI, mean fluorescence intensity. Gray shaded curve indicates isotype-matched control antibody. (n = 2 cell cultures, mean ± s.e.m., $^{*}P < 0.05$, $^{***}P < 0.005$).

*Fig. 1F- It is not clear how ubiquitination of IREB2 was induced to check the impact of OTUD1.*

[**Response**] The following effects were made to address this concern. It is known that high iron concentration induces IREB2 ubiquitination. We thus co-transfected the IREB2 along with mock, wild-type OTUD1 and its catalytically inactive mutant OTUD1 (OTUD1$^{C320S}$) into HEK293T cells. In accordance with the previous studies, the ubiquitination modification of IREB2 was increased by AFC treatment in both mock or mutant OTUD1-expressing cells (**Fig 1F**). Conversely, presence of wild-type OTUD1 remarkably inhibited iron-induced ubiquitination of IREB2 (**Fig 1F**). Our data thus demonstrate the inhibitory role of OTUD1 in regulation of IREB2 ubiquitination.

*In Fig. EV2D, why is IREB2 so intensely ubiquitinated in cells transfected with K0-Ub? All lysine residues in Ub(K0) are substituted with arginine residues, thereby Ub(K0) can only support mono-ubiquitination, but not polyubiquitination. Can authors address this anomaly?*

[**Response**] We are grateful to the reviewer for such careful review of our work. Actually, this result indicates that IREB2 undergoes with both multi-mono-ubiquitination and polyubiquitination. In the light of the role of mono-ubiquitination in transporting proteins to the lysosome where they are subsequently degraded by resident proteasomes (Kim *et al*, 2008), we thus speculate that both ubiquitin-proteasome system and autophagy-lysosome system are involved in modulation of IREB2 stability. And we will continue to study the role of OTUD1 in autophagy in our future works.

*Fig.EV2E- Can the authors show on the blot the molecular weight of Flag-IREB2? The blots seem to be lacking high molecular weight polyubiquitinated forms of Flag-IREB2 which are expected in a cell-based polyubiquitination assay. The results are less than convincing in the absence of a clear smear-like pattern typical of polyubiquitinated forms of a substrate protein.*

[**Response**] We agree that this blot seem to lack the high molecular weight polyubiquitinated forms of IREB2. We have repeated this experiment that we co-transfected IREB2 and OTUD1 into HEK293T cells in presence with AFC or DFO, respectively. As shown in **Appendix Figure S1B**, presence of OTUD1 abolished AFC-induced ubiquitination of IREB2. Our data thus demonstrate that OTUD1 deubiquitinates and stabilizes IREB2.

*Fig.1G- In the protein half-life experiment, OTUD1 levels are severely reduced by 12 hours and are almost negligible by 24 hours. However, IREB2 levels are quite stable at 12 and 24 hours. How do the authors explain this discrepancy? Moreover, it will be interesting to study how OTUD1 protein and mRNA levels are affected by low and high iron treatments.*

[**Response**] We thank the reviewer for this question. To measure the protein half-life, the band intensity was quantified with unsaturated or short exposure, which lead to undetectable OTUD1 by 24 hours. However, OTUD1 could be detected by 24 hours with long exposure, even though the expression of OTUD1 was reduced (**Fig 1G**). Besides, as mentioned above, we found that both mRNA and protein level of OTUD1 were stable in cells treated with DFO or AFC (**Response Figs 4A, B, E and F**).

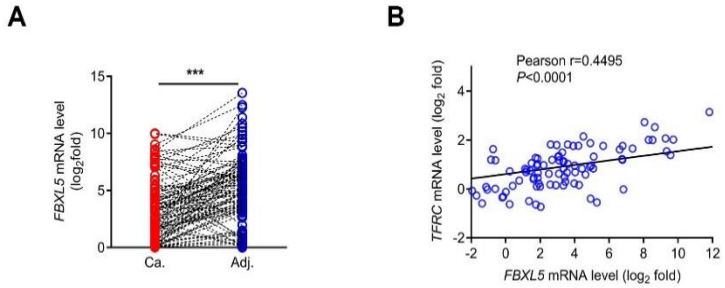
*Fig. 1I- Hemin and AFC treatments appear to affect TFRC expression in OTUD-/- cells to a very small extent, the fold difference is less than 1.5 folds. There is no significant additional impact of OTUD-/- condition on TFRC expression beyond AFC/Hemin treatments.*

[**Response**] We are grateful to the reviewer for such careful review of our work. To further ascertain the regulatory effects of OTUD1 on TFRC expression, we treated wild-type (WT) or *OTUD1*$^{-/-}$ NCM460 cells with DFO. As shown in **Fig 1I**, compared with remarkably increase of TFRC on the surface of WT cells, lower level of TFRC was induced in *OTUD1*$^{-/-}$ NCM460 cells in the treatment of DFO.

*Altogether, the results shown in Fig. 1 show that OTUD1 may have some positive effect on stability of IREB2, however fail to show convincingly the significance of this interaction on IREB2 stability under iron-regulatory conditions in comparison to the well-established IREB2-FBXL5 relationship.*

*Part 2- The authors' claim that OTUD1 mRNA expression is down-regulated in colorectal cancer tissues is well-supported by the data shown in Fig. 2. Did the authors compare and correlate mRNA expression of TFRC with that of FBXL5?*

[**Response**] As suggested, we have measured the mRNA level of *FBXL5* in primary colorectal cancer tissues as well as their matched normal tissues. In accordance with the results interrogated from TCGA database, the mRNA level of *FBXL5* was downregulated in primary colorectal cancer tissues (**Response Fig 2 and Response Fig 7A**). Additionally, we analyzed the correlation of mRNA level of *TFRC* with that of *FBXL5* in colorectal cancer tissues. As shown in **Response Figure 7B**, the expression of TFRC was positively correlated with the FBXL5, which further indicate that FBXL5 is not involved in the downregulation of TFRC in colon cancers.




**Response Figure 7. FBXL5 is downregulated in colon cancers.**
(**A**) RT–qPCR analysis of *FBXL5* mRNA levels in colon tumors and matched adjacent normal tissues (n = 101 human samples, mean ± s.e.m., ***$P < 0.001$).
(**B**) Correlation of mRNA expression of *TFRC* and *FBXL5* in colorectal cancer (n = 101 human samples, ***$P < 0.001$).

*The Figure 3 data is executed with a rigorous panel of biochemical assays and animal experiments and I greatly applaud the authors' efforts in establishing the positive impact of OTUD1 on maintenance of iron transport.*

*Part 3- the data presented in Fig. 4C-E convincingly establish that OTUD1 expression in a tumor background suppresses tumor growth and increases the survival times of animals transplanted with OTUD1-expressing cells. Additionally, it is clear from Fig. 4F, G and J that OTUD1 overexpressing tumors attract more cytotoxic T-cells to the tumor micro-environment when compared to the control tumors, alluding to involvement of OTUD1 in promoting host T-cell response against cancer.*

*Fig.5A shows mass-spectrometry based evaluation of danger-associated molecular patterns expressed in the tumor interstitial fluid. No details of the mass-spectrometric analysis have not been provided in the methods section. HSP70 and HSP90 western blots (Fig. 5B) are less than convincing since the blots are oversaturated.*

[**Response**] As suggested, we have added the mass-spectrometric specifications in the methods section in the revised manuscript. Additionally, we repeated western blot assay and quantified the level of HSP70 and HSP90 with short exposure in the tumor interstitial fluid (**Fig 6D**).

*The data in Fig. 5G-L is supportive of the authors' claim that OTUD1 is involved in ferroptosis and its overexpression in tumors sensitizes cells to ferroptosis-mediated death induced by activators such as RSL3 and erastin.*
*In the end the authors tie in together the tumor volume analysis, lymphocyte characterization and ROS/ATP measurements in wild-type or OTUD1-/-colitis-associated*

*cancer model. It is clear from data presented in Fig. 6B and C that OTUD1 expression is negatively correlated with tumor load in animals. Further, authors satisfactorily show that through Fig. 6E that OTUD1 expression in tumors is positively correlated with maintenance of TFRC expression. Comparisons of levels of intracellular ROS and ATP leakage in the tumor interstitial fluid between wild-type and OTUD1-/- animals argue for a role of OTUD1 in development of colon cancer.*

*Other critiques related to manuscript structure:*
*The general structure of the manuscript lacks coherence, lucidity and succinctness, which makes it difficult to comprehend the reasoning and relevance of the experiment performed and conclusions drawn from the text alone. The prevalence of easily avoidable typos makes it hard for the reader to appreciate the reason, logic and importance of experiments and observations reported in the manuscript. The manuscript requires thorough editing both in terms of the language as well as scientific accuracy of the conclusions drawn from the observations.*
*There are lots of typos throughout the text, some examples are mentioned below:*
*Section "OTUD1 acts as a deubiquitinase of IREB2'- "...supplementation of Deferoxamine (DFO), the iron chelation,.."*
*Abstract- "..In spite of the essential element for life,...."*
*Discussion- "...there was no spontaneous tumor developed in Otud1-/- mice within one year old."*

*The abstract is poorly constructed with insufficient background information provided to build a case for lack of current understanding of role of iron in cancer-related immune response and how the authors' work contributes to filling these gaps. Importantly, the authors have failed to cite most recent and comprehensive reviews relevant to their research interest such as 2018 Annual review of nutrition "Iron and Cancer" published from Torti lab.*

*References:*
*1. Vashisht AA, Zumbrennen KB, Huang X, et al. Control of iron homeostasis by an iron-regulated ubiquitin ligase. Science. 2009;326(5953):718-721.*
*2. Salahudeen AA, Thompson JW, Ruiz JC, et al. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. Science. 2009;326(5953):722-726. doi:10.1126/science.1176326*
*3. Suzy V. Torti, David H. Manz, Bibbin T. Paul, et al. Iron and Cancer. Annual Review of Nutrition. 2018 38:1, 97-125.*

[**Response**] Thank you for these insightful comments and suggestions. As suggested, we have thoroughly revised our manuscript and corrected the typos. And we have also cited “Suzy *et al*, 2018” in the introduction section.

References:
1. Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. Ubiquitin signals

autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci U S A. 2008 105(52):20567-74.

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*Referee #3:*

*This manuscript reports: (1) OTUD1 deubiquitinates and stabilizes IREB2, promoting TFRC-mediated iron transportation. (2) OTUD1 increases cellular iron uptake that triggers immunogenic cell death, leading to suppression of tumor growth. (3) Loss of OTUD1 impedes tumor-reactive T cell accumulation and promotes colon cancer progression. These findings are very interesting and the authors provided data to support their conclusions. The manuscript will be suitable for publication in EMBO reports if the authors can provide data to address the following concerns and strengthen their conclusions:*

*1. In vitro deubiquitination assays, with wild-type and catalytically inactive (C320S) OTUD1, should be used to demonstrate that OTUD1 can deubiquitinate IREB2 directly.*

[**Response**] As suggested, we performed the *in vitro* deubiquitination assay with wild-type and catalytically inactive (C320S) OTUD1. As shown in **Figure EV2E**, OTUD1 rather than its catalytically inactive (C320S) mutant directly deubiquitinated IREB2, which further confirms the role of OTUD1 in regulation of IREB2.

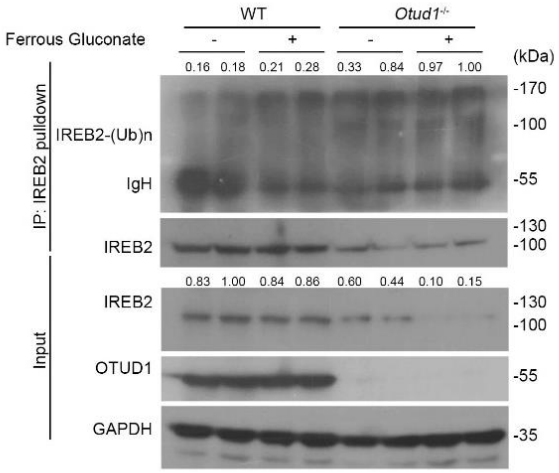
*2. Figure 1F (in vivo deubiquitination assays): how to confirm that the Ub signal is indeed from IREB2, but not from IREB2-interacting proteins?*

[**Response**] Thank the reviewer for this question. To further confirm that the ubiquitination modification is indeed from IREB2 rather than other IREB2-associated proteins, we thus co-transfected the IREB2 along with mock, wild-type OTUD1 and catalytically inactive mutant OTUD1 into HEK293T cells. Following the treatment of AFC, the ubiquitination modification of IREB2 was increased in both mock or mutant OTUD1-expressing cells (**Fig 1F**). Conversely, presence of wild-type OTUD1 remarkably inhibited IREB2 ubiquitination (**Fig 1F**). Our data thus demonstrate that OTUD1 restricts iron-induced IREB2 ubiquitination.

*3. Is polyubiquitination of Ireb2 upregulated in Otud1-knockout mouse tissues?*

[**Response**] Yes. In order to test whether ubiquitination of IREB2 is upregulated in *Otud1*$^{-/-}$ mouse colons, we intragastrically administrated either Ferrous Gluconate or saline into wild-type or *Otud1*$^{-/-}$ mice. 24hrs later, we used anti-IREB2 antibody to pulldown endogenous IREB2 from the lysates of wild-type (WT) or *Otud1*$^{-/-}$ colon tissues. We then used anti-ubiquitin antibody to assess the ubiquitination modification of IREB2. In accordance with the cell line data that loss of OTUD1 suppressed IREB2 expression, protein levels of IREB2 were decreased in *Otud1*$^{-/-}$ mice colon tissues as compared with those in WT controls (**Response Fig 8**). Moreover,

supplementation of Ferrous Gluconate promoted IREB2 ubiquitination and suppressed IREB2 expression in both wild-type and *Otud1*$^{-/-}$ colon tissues (**Response Fig 8**). Because of less amount of IREB2 in *Otud1*$^{-/-}$ colon tissues, the ubiquitination of IREB2 exhibited slightly increase in *Otud1*$^{-/-}$ colon tissues as compared with those wild-type colon tissues (**Response Fig 8**).



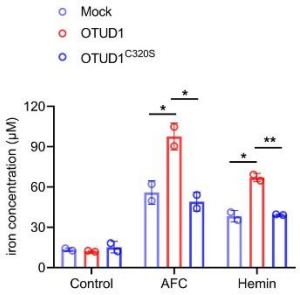

**Response Figure 8. Loss of OTUD1 promotes IREB2 ubiquitination and degradation.**
Wild-type (WT) and *Otud1*$^{-/-}$ mice were intragastrically administrated with either Ferrous Gluconate or saline, respectively. 24hrs later, colon tissue lysates were immunoprecipitated with anti-IREB2 antibody and analyzed by immunoblot with anti-IREB2, anti-ubiquitin and anti-GAPDH antibodies. The expression of indicated proteins were measure by western blot and the band intensity was quantified by *Image J* software.

*4. Figure 3: rescue experiments and/or gain-of-function experiments, with the C320S mutant as the negative control, should be presented to demonstrate that OTUD1 promotes cellular iron uptake. Also, the authors should demonstrate that IREB2 mediates the role of OTUD1 in iron uptake.*

[**Response**] As suggested, we stably expressed mock, OTUD1 or its catalytically inactive (C320S) mutant vector in CT26 cells and assessed the cellular capacity of iron transportation. As shown in **Response Fig 9**, in treatment with ammonium ferric citrate (AFC) or Hemin, enforced expression of OTUD1 rather than its catalytically inactive mutant enhanced iron uptake as compared with mock did.

To test whether the stimulatory effect of OTUD1 on iron absorption is in an IREB2-dependent manner, we used CRISPR-Cas9 to delete the *Ireb2* in CT26 cells. As shown in **Figure EV3A**, compared with WT cells, the presence of OTUD1 exerts little effects on iron absorption in *Ireb2*$^{-/-}$ cells treated with AFC. Our data thus demonstrate that OTUD1 promotes IREB2-TFRC signaling and drives iron uptake

through its deubiquitinating activity.




**Response Figure 9. OTUD1 rather than OTUD1$^{C320S}$ promotes iron transportation.**
Intracellular iron concentration was measured in CT26 cells stably expressing mock, OTUD1 or OTUD1$^{C320S}$ treated with AFC and Hemin (n = 2 cell cultures, mean ± s.e.m., *$P$ < 0.05, **$P$ = 0.0065).

*5. Figures 4 & 5: the C320S mutant should be included as a negative control in these experiments. Can IREB2 knockdown reverse the effects of OTUD1 overexpression?*

[**Response**] As suggested, we have added the C320S mutant as a negative control in these experiments (**Figs 4A, B, F, G, I, J** and **Figs 5D** and **Fig 6D**). To test whether the regulatory effects of OTUD1 on iron uptake is in an IREB2-dependent manner, we used CRISPR-Cas9 to delete the *Ireb2* in CT26 cells. Both western blot and flow cytometry assays revealed that overexpression of OTUD1 promoted TFRC expression in wild-type (WT) CT26 cells rather than *Ireb2*$^{-/-}$ cells (**Figs EV2G** and **H,** and **Appendix Fig S2C**). Accordingly, compared with WT cells, the presence of OTUD1 exerts little effects on iron absorption in *Ireb2*$^{-/-}$ cells treated with AFC (**Fig EV3A**). Collectively, our data demonstrate that IREB2 is required for the modulation of iron uptake by OTUD1.

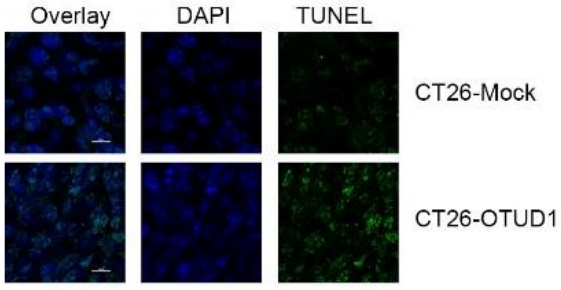
*6. In those in vivo tumor growth experiments, the levels of ferroptosis, iron, lipid ROS should be examined and compared between different groups. This will be needed to prove that OTUD1 drives intracellular iron accumulation and promotes oxidative damage, which in turn augments ferroptosis.*

[**Response**] We are grateful for the reviewer's suggestions. In order to ascertain that ferroptosis mainly contributes to the tumor-suppressive function of OTUD1, we used vitamin E (VE) that is radical scavenger, to suppress ferroptosis commitment. Considering only a portion of VE dosage can reach the tumor by oral administration and its water insoluble characteristic, we exploited a molecular-matched strategy to prepare VE-loading TPGS nanoparticles (NP-VE) with extremely high drug loading levels (up to 10 mg/ml). The nanoparticles have particle sizes of 144.37±0.39 nm and

zeta potentials of -29.43±0.46 mV determined by dynamic light scattering (DLS) methods (**Figs EV4D** and **E**). *In vitro* assay showed that NP-VE treatment curtailed the RSL3-induced ferroptosis in OTUD1-expressing cells (**Fig EV4F**).

We next intratumorally injected with NP-VE into mice bearing mock-expressing or OTUD1-expressing tumors (**Fig 5H**). As shown in **Figure 5I**, in contrast to the little effects on tumor growth in mice bearing mock-expressing tumor, NP-VE treatment blocked the inhibitory effects of OTUD1 on tumor growth. Moreover, flow cytometry analysis revealed that NP-VE treatment remarkably reduced the intracellular level of ROS in both mock and OTUD1-expressing tumors (**Fig 5J** and **Appendix Fig S6C**). Notably, supplementation of NP-VE also reduced the ratio of $CD8^+$ tumor-infiltrated T cell (**Fig 5K** and **Appendix Fig S6D**).

As suggested, we also measured the levels of cell death, iron concentration and ROS in tumors expressing mock, OTUD1 or its catalytically inactive mutant. As shown in **Figure 5G** and **Appendix Figure S6B**, compared with mock or $OTUD1^{C320S}$-expressing tumors, higher level of ROS was detected in OTUD1-expressing tumors. Consistently, through analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) assay, we found that intracellular iron concentration was remarkably increased in OTUD1-expressing tumors as compared with that in mock or $OTUD1^{C320S}$-expressing tumors (**Fig 4I**). Additionally, we also used TUNEL staining to assess the ratio of cell death. As shown in **Response Figure 10**, higher proportion of TUNEL positive cells were detected in OTUD1-expressing tumors. Collectively, our data thus demonstrate that the stimulatory role of OTUD1 in ferroptosis drives host antitumor immunity and suppresses tumor development.




**Response Figure 10. OTUD1 augments cell death *in vivo*.**
Mock or OTUD1-expressing tumor sections were stained with TUNEL, followed by evaluating with fluorescence microscopy. Nuclear was indicated by DAPI. The scale bars represent 10 μm.

*7. Statistical analysis is lacking in Figure 2E.*

[**Response**] As suggested, we have added statistical analysis of Figure 2E in the Table 1.

*8. The English of this manuscript needs editing.*

[**Response**] As suggested, we have thoroughly revised our manuscript. Thank you again for these insightful comments and critical suggestions.

## 1st Revision - Editorial Decision 1st Dec 2020

Dear Dr. You,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your paper in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- There are two authors named YY in the author contributions. Please distinguish these, e.g. using YueY and YuxY.

- Presently, the scale bars in the microscopic images are not uniform, and most are too small/thin to be clearly visible online. Please add scale bars of similar style and thickness to all the images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures). Please provide statistical testing where applicable.

- In the diagrams shown in Fig. 3A and 5F you show error bars, although you indicate in the legend that only two replicates are shown. Please show these data without statistics, by showing the two dataset separated. This is much more transparent, and illustrates better the data. Or add a third replicate to do proper statistics.

- Please change the labeling 'Untreatment' in Figs. 6A/B to 'Untreated' or 'Control'.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done. Please note that these checks were done on the original version of your revised manuscript. Please do NOT use this file for preparing the final revised version, as it does not contain those changes done before the paper was sent for re-review (e.g. regarding data availability, or replicates n=2).

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

---
Referee #1:

The authors have addressed the concerns raised during the first revision process and the paper is substantially improved. Congratulations.

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Referee #2:

The manuscript entitled "The deubiquitinase OTUD1 enhances iron transportation and potentiates host antitumor immunity" by Song et al, reports molecular mechanisms underlying regulation of IRE-binding protein IREB2 by deubiquitinase OTUD1 and the relevance of the pathway in colorectal cancer development. The revised manuscript submitted by the authors has satisfactorily addressed the most important critiques raised by the reviewers earlier. In the revised version of the work, the authors have extensively investigated the role of FBXL5, the E3 ubiquitin ligase which is critical for IREB2 regulation, in OTUD1-IREB2 axis. The experiments performed and pieces of evidence provided in the point-by-point response version to address queries raised about the plausible involvement of FBXL5 in the OTUD1-mediated regulation of IREB2 sufficiently demonstrate that the regulation of IREB2 by OTUD1 is primarily FBXL5-independent in the context of colorectal cancer setting. Hence, the manuscript is suitable for publication in its current form.

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Referee #3:

The authors have addressed my previous comments. I have no additional concerns.

The authors have addressed all minor editorial requests.

## 2nd Revision - Editorial Decision 3rd Dec 2020

Fuping You
Peking University Health Science Center
No. 38, Xueyuan Rd, Haidian District
Beijing 100191
China

Dear Dr. You,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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## Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 $\chi 2$ 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)

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|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Sample size was chosen based on the standard practices in the field. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | Sample size was chosen based on the standard practices in the field. No statistical methods were used to predetermine the sample size. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No data were excluded from the analysis. |
| 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. | Yes, we used randomization procedure to minimize the effects of subjetiive bias. |
| For animal studies, include a statement about randomization even if no randomization was used. | For animal studies, randomization was performed. |
| 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. | Yes, we employed blinding of the the investigator method to minimize the effects of subjective bias during group allocation when assessing the resutls. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Animal studies were not blinded. |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, the statistical tests we used to analyze figures are justifies as appropriate. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | All the data meet the assumptions of the tests. Statistical analysis was performed using GraphPad Prism software v7.0 (GraphPad Prism, RRID: SCR_002798). Differences between two groups were calculated using a two-tailed Student's t test. In tumor transplantation experiments, mice survival status was analyzed by Log-rank (Mantel-Cox) test. |
| Is there an estimate of variation within each group of data? | Yes, variation within each group was estimated. |

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|---|---|
| Is the variance similar between the groups that are being statistically compared? | Yes, the variance was similar between the groups that are being statistically compared. |

## C- Reagents

| | |
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| 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). | The antibodies used in this study were as follows: anti-IREB2 (Proteintech, 23829-1-AP), anti-OTUD1 (abcam, ab122481), anti-TFRC (abcam, ab214039), anti-FBXL5 (Abclonal, A5602), anti-Ub (FK2) (LifeSensors, LSS-AB120), anti-FLAG (Sigma, F3165), anti-GFP (RayAntibody, RM1008), anti-HA (Sigma, H3663), anti-GAPDH (RayAntibody, RM2002), anti-CD3 (biolegend, 145-2C11), anti-CD4 (eBioscience, GK1.5), anti-CD8 (eBioscience, 53-6.7), anti-CD45 (eBioscience, 30-F11), anti-mouse TFRC (biolegend, RI7217), anti-human TFRC (biolegend, CY1G4). |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | All the cell lines including HEK293T, Lewis lung carcinoma (LLC) and CT26 in this study were obtained from American Type Culture Collection (ATCC).None of the cell lines were authenticated.All of the cell lines were negative for mycoplasma contamination. |

* 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. | C57BL/6 and BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed and maintained under specific pathogen-free conditions. Background-, gender- and age-matched mice were used in the experiments. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | All animal experiments were performed in accordance with protocols approved by the Ethics Committee of Peking University Health Science Center(approved number LA2016240). |
| 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. | For animal studies, ARRIVE guidelines were followed. |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | All procedures were conducted under the approval of the Ethics Committee of Peking University Health Science Center. |
| 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. | Informed patient consent was obtained from all subjects in advance of sample collection (in accordance with the Helsinki Declaration). |
| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | The data that support the findings in this study are available from the corresponding author upon reasonable requests. The RNA-seq data that support the findings in this study have been deposited in the GEO database with the accession codes GSE140059 and GSE140058. |
| 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

| | |
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| 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. | The study does not have the concern of dual use. |