EMBO reports

OMA1 reprograms metabolism under hypoxia to promote colorectal cancer development

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1st Editorial Decision 12th Jun 2020

Dear Dr. Song,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

We concur with the referees that the proposed role of OMA1 in colorectal cancer development is in principle very interesting. However, referees also raise important concerns that need to be addressed to consider publication here.

In particular, referees, especially referee #1, are not convinced by the causality between some findings - such as depletion of OMA1 and respiratory complex assembly, as well as OMA1 depletion and glycolysis. Similarly, referee #2 requires better support into the role of HIF1alpha in the effect of OMA1 on the increase in glycolytic flux and the role of ROS in OMA1 mediated HIF1 alpha stabilization.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Of note, referee #1 also makes the following remark 'How OMA1 supports ROS production remains enigmatic'. Even if this question cannot be addressed experimentally, it is important to discuss this.

Should you be able to address all criticisms in full, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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

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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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1. A data availability section providing access to data deposited in public databases is missing (where applicable).

2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

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. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
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4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be

bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

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7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) 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://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

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 & Method) 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 (and computer code) 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])

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10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Wu et al. provide evidence for a role of regulated mitochondrial proteolysis in colorectal tumorigenesis. They show that the inner mitochondrial membrane protease OMA1 is required for normal tumour growth in a mouse model of inflammatory colorectal cancer and for tumour cell subcutaneous xenograft growth. They suggest that OMA1 plays a role in cancer cell metabolic adaptation since they observe enhanced OMA1-meditated proteolysis in tumours and in cells under hypoxia, supporting the notion that OMA1 activity contributes to tumour cell adaptation. They also identify novel putative OMA1 substrates via interaction-based proteomics and reveal that OMA1 processes/degrades components of the electron transport chain.

The observation that OMA1 limits colorectal cancer development in an AOM/DSS is intriguing and of relevance to the study of mitochondrial regulation during tumorigenesis. When it comes to why OMA1 is important for colorectal cancer development however, I feel that the findings are overinterpreted, often based on correlative evidence only, leading to rather vague descriptions of OMA1's broad contribution to cancer metabolism. For instance, the accumulation of respiratory chain complexes in OMA1-deficient colorectal cancer cells does not allow to conclude that OMA1 promotes the disassembly of these complexes. In general, the data are insufficient to support the proposed model of OMA1 being "coordinating glycolysis and oxidative phosphorylation" and "promoting the Warburg effect" to facilitate colorectal cancer (CRC). The authors do not address whether the mild glycolytic deficiency, or reduced ROS and hypoxic signalling, or accumulation of

OXPHOS substrates observed in OMA1 KO cells has any bearing on colon cancer cell phenotypes or CRC development.

Other comments:

The AOM/DSS chemical approach is a useful method to relatively quickly induce inflammatory CRC. The authors note that OMA1 KO are less susceptible to colitis induced by the course of DSS (Fig. 1 B,C,D). A reduced inflammatory response in OMA1 KO will have a bearing on tumour development and likely contributes to the impressive reduction of tumour size and number in these animals. Since the xenograft experiments nicely support a more direct role for OMA1 in CRC development, I think it would be required for the authors to at least remark that OMA1 regulation of inflammation could contribute to the CRC pathology.

The argument that the OMA1-OPA1 axis is required to regulate glycolysis under hypoxia is very confused. I do not see any evidence that OMA1-mediated processing of OPA1 per se is required for the glycolytic response. The authors interpret that OMA1-processing of OPA1 is important during hypoxia simply because OPA1-KO cells are susceptible to glycolysis inhibition (Fig. 4 EV4D,E, Fig. 2 E-H). Furthermore, the authors should cite previous work showing that OPA1 processing is enhanced in hypoxia (e.g An HJ et al., PNAS 2013). OPA1 total levels are also limited by YME1L during hypoxia (MacVicar et al., Nature 2019), which may explain why total OPA1 levels appear reduced in OMA1 KO tumours here (Fig. EV5 C and D). The OMA1-dependent destruction of tumour cell cristae shown in Fig. EV5 is striking but whether this is an indirect consequence of impaired tumor development or how OPA1 processing by OMA1 fits into the final model is not clear.

The requirement of OMA1 for HIF1a stabilization is striking and the authors explain this by reduced ROS formation in the absence of OMA1. However, how OMA1 support ROS formation remains enigmatic.

The identification of novel substrates should be more directly confirmed using chase experiments upon cycloheximide treatment or in hypoxic conditions in WT, OMA1 KO and OMA1 KO cells complemented with WT-OMA1/proteolytic inactive OMA1.

Data quality is generally high but there are some points to be made. Representative blots are used throughout without quantification. The authors should at least indicate the number of experiments performed. White gaps in western blots of potential OMA1 substrates between WT and OMA1-KO lanes e.g EV6 B and 4F should be referred to, which could strongly affect the interpretation.

Except for panel G, the contribution of Fig.5 to the paper's principle message is unclear and unhelpful. Without quantification, molecular weight markers and SDS-PAGE loading controls, the BN-PAGEs in A and B cannot really be interpreted.

Bioinformatic analysis reveals that OMA1 mRNA expression in enhanced in human tumours but this does not appear to be the mechanism that regulates its activity in hypoxic cells (Fig. EV2), perhaps the authors can speculate on how OMA1 is stimulated during hypoxia.

The authors do not provide evidence for autocatalysis of OMA1 in the tumour situation or hypoxia, which they describe as a potential "stop button" in hypoxic adaptation. Previous work has shown that OMA1 is degraded by YME1L in hypoxia (MacVicar et al., 2019 Nature). To conclusively test OMA1 autocatalysis in colon cancer cells, the authors could see whether proteolytic-dead OMA1 is degraded upon hypoxia in OMA1 KO cells.

Method/reference for the HCT116 OMA1 KO cell line is missing

Referee #2:

In this study, Wu and colleagues investigate the role of OMA1 protease in metabolic rewiring and its relationship in cancer. Starting from showing a correlation between OMA1 expression and colorectal tumour development they progress to show that loss of OMA1 inhibits tumourigenesis in two models (AOM/DSS, xenograft). They then show that OMA1 promotes HIFalpha expression underhypoxia, linking to the effects of glycolysis described earlier. Investigating the mechanistic basis for this, they describe that under conditions of hypoxia or CCCP uncoupler, OMA1 cleaves various respiratory chain complex proteins, affecting complex levels, providing a means of metabolic rewiring towards a glycolytic phenotype. The study is well-performed, dataset extensive and largely supports the authors' conclusions. I have a few points outstanding that should be addressed.

- In my opinion, based on the authors data, how OMA1 promotes a shift to glycolysis, may be multifaceted - potentially by HIF1 alpha upregulation but equally via degradation of respiratory complex proteins - the importance of the latter is difficult to define (given that many substrates are involved), however the importance of HIF1 alpha to OMA1 mediated rewiring (i.e. shift to glycolysis) should be readily testable by inhibiting HIF1 alpha expression.

- Extending on this point, the authors state (Fig 3) that ROS generated by OMA1 is required for HIF1 lpaga upregulation - this is not directly tested (i.e. through use of ROS scavenger), either the claim should be moderated somewhat, or an expt. to test this should be performed.

- The error bars on some of the metabolic assays (e.g. Fig 2) appear impossibbly small to represent biological replicates, are they instead technical replicates ?

- The ms. is data rich, I do realise journal figure limits etc. but ideally the intial results section should be presented in figure 1 (not various extended figures). The grammar, flow of the text can also be improved to help the reader.

Referee #3:

The work by Wu et al describes the involvement of OMA1 in the progression and generation of colorectal cancer. The data shown are interesting and improve our understanding of the mechanisms linking hypoxia and metabolic reprogramming within the tumor microenvironment. However, some points need to be addressed before accepting the main conclusions of the paper:

Major points:
The authors report that OMA1 is activated under hypoxia, while its total levels do not change. This may indicate that OMA1 is activated post-translationally. Several works (Simula et al., 2020 - Jiang et al., 2014 PNAS and many others) indicate that active OMA1 can be detected as a shorter fragment compared to full-length protein. Authors should check.
Data in Figures 2A-D are interpreted using student t-test. However, since there are more than 2 samples in each experimental layout, an ANOVA (two-ways) should be performed instead (also because the differences are very little and must be properly statistically tested).
Western blot images in Figures 3A, 3B, 4F, 5A-B and EV5C should be accompanied by a graph indicating the quantification of the bands. In addition, image 5B seems to show that OMA1-KO is not able to restore complexes I-IV under hypoxia compared to control hypoxia conditions, while the authors state the opposite. Can the Authors clarify please? The image does not support author conclusions. Different representative images should be chosen and adding quantification may help.
In figure EV5C the rescue effect on Opa1 due to OMA1-KO is not evident.
Authors state that OMA1 regulates HIF1a (and then glycolysis) through ROS production. However, a rescue experiment showing that restoration of ROS in OMA1-KO cells does increase HIF1 (and glycolysis) is missing. As shown, the mechanism lacks an important step to be firmly established.

Minor Points:
It is not clear what the asterisks indicate in Fig 1B. How was the statistics calculated? ANOVA TW should be performed.
A western blot showing the over-expression of Flag-OMA1 related to Figure 2 should be shown at least in supplementary.
The experimental setup to induce hypoxia in vitro should be better explained in the Methods section.

We sincerely thank the reviewers for valuable comments and constructive criticisms, which were of great help in revising the manuscript. According to the reviewers' comments and suggestions, the revised manuscript has been systematically improved with new data and additional interpretations. Reviewers' comments are responded point-by-point as bellow. Reviewers' points are underlined and italic for easier reference. Descriptions for all newly performed experimental results and other changes are highlighted in blue in the revised manuscript.

Referee #1:

Wu et al. provide evidence for a role of regulated mitochondrial proteolysis in colorectal tumorigenesis. They show that the inner mitochondrial membrane protease OMA1 is required for normal tumour growth in a mouse model of inflammatory colorectal cancer and for tumour cell subcutaneous xenograft growth. They suggest that OMA1 plays a role in cancer cell metabolic adaptation since they observe enhanced OMA1-meditated proteolysis in tumours and in cells under hypoxia, supporting the notion that OMA1 activity contributes to tumour cell adaptation. They also identify novel putative OMA1 substrates via interaction-based proteomics and reveal that OMA1 processes/degrades components of the electron transport chain.

The observation that OMA1 limits colorectal cancer development in an AOM/DSS is intriguing and of relevance to the study of mitochondrial regulation during tumorigenesis. When it comes to why OMA1 is important for colorectal cancer development however, I feel that the findings are overinterpreted, often based on correlative evidence only, leading to rather vague descriptions of OMA1's broad contribution to cancer metabolism. For instance, the accumulation of respiratory chain complexes in OMA1-deficient colorectal cancer cells does not allow to conclude that OMA1 promotes the disassembly of these complexes.

We appreciate the reviewer's comments and thoughtful critiques. To investigate the role of OMA1 in the regulation of respiratory chain complexes, we performed additional experiments in the revised manuscript. Recent literature evidence suggest that mutations in NDUFA4 result in the dysfunction of cytochrome c oxidase (Pitceathly Rd, 2013), and COX4L1 is associated with COX1 translation (Richter-Dennerlein et al, 2016). Furthermore, NDUFB5 and NDUFB6 are the subunits of NADH dehydrogenase (Loublier et al, 2011; Stroud et al, 2016). In our manuscript, we identified that mitochondrial respiratory chain complexes components

NDUFB5, NDUFB6, COX4L1 and NDUFA4 are proteolytic substrates of OMA1 (Fig 5 of the revised manuscript). We also assessed the integrity of mitochondrial respiratory chain complexes in COX4L1, NDUFB6 or NDUFB5 knockdown, or NDUFA4 KO cells by blue native PAGE in the revised manuscript. As shown in Fig EV4E of the revised manuscript (new data), NDUFB5 or NDUFB6 knockdown in HCT116 cells led to a significant decrease of complex I, and COX4L1 knockdown or NDUFA4 knockout results in a reduction of complex IV. These results demonstrate that OMA1-mediated processing or degradation of NDUFB5, NDUFB6, COX4L1 and NDUFA4 in hypoxia impairs the assembly of mitochondrial respiratory chain complexes.

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In general, the data are insufficient to support the proposed model of OMA1 being "coordinating glycolysis and oxidative phosphorylation" and "promoting the Warburg effect" to facilitate colorectal cancer (CRC).

We thank the reviewer for the comments. To further support this proposed model, we performed some experiments in the revised manuscript. Since NDUFB5, NDUFB6, COX4L1, and NDUFA4 are important components of mitochondrial oxidative phosphorylation (OXPHOS) complexes (Balsa et al, 2012; Böttinger et al, 2013; Loublier et al, 2011; Pitceathly Rd, 2013; Richter-Dennerlein et al, 2016), and are degraded or processed by activated-OMA1 (Fig 5 of the revised manuscript), we explored the glycolytic metabolism in cells lacking one of these substrates. As shown in Fig EV5C and D of the revised manuscript (new data), we observed significantly increased levels of glucose uptake and lactate production in shNDUFB5 (NDUFB5 knockdown), shNDUFB6 (NDUFB6 knockdown), shCOX4L1 (COX4L1

knockdown) or NDUFA4 knockout (KO) cells compared with control cells, suggesting that the depletion of these OMA1 substrates not only inhibits oxidative phosphorylation but also promotes glycolysis. In addition, the protein levels of OMA1 substrates including NDUFB5, NDUFB6, COX4L1, and NDUFA4 in tumor tissues were remarkably decreased (degraded) compared with that in adjacent normal tissue in WT mice, but OMA1 KO inhibited the degradation (Fig 6C of the revised manuscript), indicating that OMA1 deficiency prevents the up-regulation of glycolysis and down-regulation of oxidative phosphorylation are probably due to the inhibition of the degradation of OMA1 substrates. Furthermore, several studies have reported that enhanced glycolysis promotes colorectal cancer (Nie et al, 2020; Pate et al, 2014; Tang et al, 2019). In our manuscript, under hypoxia, OMA1 KO decreases ROS production to prevent the upregulation of HIF-1α (Fig 4 of the revised manuscript), a key regulator of glycolytic metabolism in cancer, indicating that OMA1 regulates glycolysis in a HIF-1α dependent manner. Overall, these data indicate that OMA1 KO prevents colorectal cancer development by inhibiting the increase of HIF-1α and the degradation of OMA1 substrates, which contribute to the shift from oxidative phosphorylation to glycolysis.

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Nie H, Ju H, Fan J, Shi X, Cheng Y, Cang X, Zheng Z, Duan X, Yi W (2020) O-GlcNAcylation of PGK1 coordinates glycolysis and TCA cycle to promote tumor growth. *Nat Commun* 11: 36

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Pitceathly Rd RSWY (2013) NDUFA4 Mutations Underlie Dysfunction of a Cytochrome c Oxidase Subunit Linked to Human Neurological Disease. *Cell Reports* 3: 1795–1805

Richter-Dennerlein R, Oeljeklaus S, Lorenzi I, Ronsor C, Bareth B, Schendzielorz AB, Wang C, Warscheid B, Rehling P, Dennerlein S (2016) Mitochondrial Protein Synthesis Adapts to Influx of Nuclear-Encoded Protein. *Cell* 167: 471-483 e410

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The authors do not address whether the mild glycolytic deficiency, or reduced ROS and hypoxic signalling, or accumulation of OXPHOS substrates observed in OMA1 KO cells has any bearing on colon cancer cell phenotypes or CRC development.

We are grateful to the reviewer for constructive criticisms. Several genomic, proteomic and metabolomic studies have revealed metabolic alteration in colorectal cancer including elevated tissue hypoxia, enhanced glycolytic metabolism, nucleotide biosynthesis, lipid and steroid metabolism (Bi et al, 2006; Chan et al, 2009; Muzny et al, 2012; Vasaikar et al, 2019). Glycolysis produces energy and the glycolytic intermediates to promote growth, proliferation, and survival for cancer cells. Previous studies have indicated that glycolytic metabolism drives colorectal cancer (CRC) initiation and progression, and increased glycolysis in the colorectal cancer is also associated with immune suppression (La Vecchia & Sebastian, 2020; Vasaikar et al, 2019). And inhibition of glycolysis is shown to be an effective method to decrease the proliferation of colorectal cancer cells and inhibit the growth of CRC tumors in vivo (Nie et al, 2020; Pate et al, 2014; Sebastian et al, 2012). In addition, reactive oxygen species (ROS) produced during hypoxia plays a critical role in stabilizing and activating HIF-1α (Movafagh et al, 2015; Niecknig et al, 2012), which is a mediator of glycolytic metabolism. Consistently, several studies have reported that reducing ROS level and hypoxic signaling play an important role in preventing colorectal cancer (Bell et al, 2011; Myant et al, 2013). In our manuscript, the deficiency of OMA1 substrates including NDUFB5, NDUFB6, COX4L1, or NDUFA4 caused the disassembly of mitochondrial respiratory chain complexes (Fig EV4E of the revised manuscript, new data), increased the generation of mtROS (Fig EV5A and B of the revised manuscript, new data) and promoted a shift to glycolysis (Fig EV5C and D of the revised manuscript, new data). Moreover, OMA1 KO inhibits the CRC development (Fig 2A-H of the revised manuscript). Therefore, based on the published data and our data in this manuscript, the mild glycolytic deficiency, reduced ROS and hypoxic signaling, and accumulation of OXPHOS substrates in OMA1 KO cells contribute to the inhibition of CRC development.

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Vasaikar S, Huang C, Wang X, Petyuk VA, Savage SR, Wen B, Dou Y, Zhang Y, Shi Z, Arshad OA et al (2019) Proteogenomic Analysis of Human Colon Cancer Reveals New Therapeutic Opportunities. *Cell* 177: 1035-1049 e1019

Other comments:

The AOM/DSS chemical approach is a useful method to relatively quickly induce inflammatory CRC. The authors note that OMA1 KO are less susceptible to colitis induced by the course of DSS (Fig. 1 B,C,D). A reduced inflammatory response in OMA1 KO will have a bearing on tumour development and likely contributes to the impressive reduction of tumour size and number in these animals. Since the xenograft experiments nicely support a more direct role for OMA1 in CRC development, I think it would be required for the authors to at least remark that OMA1 regulation of inflammation could contribute to the CRC pathology.

We sincerely thank the reviewer's comments. As suggested by the reviewer, we performed the qRT-PCR assay to analyze the relative mRNA levels of the pro-inflammatory genes (TNFα, IL-6, COX-2, CCL2) in the intestines of WT or OMA1 KO mice treated with AOM/DSS. qRT-

PCR data revealed that after treatment of AOM/DSS, the mRNA levels of TNFα, IL-6, COX-2, and CCL2 in the intestines of OMA1 KO mice were significantly reduced compared with that of WT mice (Fig 2J of the revised manuscript, new data), suggesting that OMA1 regulates AOM/DSS-induced inflammation, which is associated with AOM/DSS-induced colorectal cancer. We have added these data and remarked the role of OMA1-regulated inflammation in CRC pathology in the revised manuscript.

The argument that the OMA1-OPA1 axis is required to regulate glycolysis under hypoxia is very confused. I do not see any evidence that OMA1-mediated processing of OPA1 per se is required for the glycolytic response. The authors interpret that OMA1-processing of OPA1 is important during hypoxia simply because OPA1-KO cells are susceptible to glycolysis inhibition (Fig. 4 EV4D,E, Fig. 2 E-H). Furthermore, the authors should cite previous work showing that OPA1 processing is enhanced in hypoxia (e.g An HJ et al., PNAS 2013). OPA1 total levels are also limited by YME1L during hypoxia (MacVicar et al., Nature 2019), which may explain why total OPA1 levels appear reduced in OMA1 KO tumours here (Fig. EV5 C and D). The OMA1-dependent destruction of tumour cell cristae shown in Fig. EV5 is striking but whether this is an indirect consequence of impaired tumor development or how OPA1 processing by OMA1 fits into the final model is not clear.

We appreciate the reviewer for his/her constructive comments and suggestions. Mitochondrial protease OMA1 and Yme1L regulate OPA1 processing and degradation under certain stresses (Anand et al, 2014). Under hypoxia, OPA1 processing is enhanced (An HJ et al., 2013), and the OPA1 level is regulated by Yme1L (MacVicar et al., 2019) (these two papers have been cited in the revised manuscript). In our manuscript, we further investigated the role of OMA1 and Yme1L in OPA1 processing under hypoxia, and found that both OMA1 and Yme1L contribute to OPA1 processing and degradation under hypoxia (Fig EV3G of the revised manuscript, new data), which explain why total OPA1 levels are reduced in OMA1 KO tumors (Fig EV3C of the revised manuscript). Therefore, both Yme1L and OMA1 regulate OPA1 processing and degradation under hypoxia, which also promotes glycolysis under hypoxia. We have added and discussed these data in the revised manuscript.

Besides acting as a key factor of mitochondrial inner membrane fusion, OPA1 is required for mitochondrial cristae formation (DeVay et al., 2009; Patten et al., 2014; Song et al., 2009). We found that OMA1 KO inhibits mitochondrial cristae loss in mice colorectal tumors (Fig

EV3A and B of the revised manuscript), furthermore, OMA1 KO inhibited the degradation of OPA1 in the colorectal tumors (Fig EV3C of the revised manuscript). These results indicate that OMA1-mediated OPA1 processing or degradation is associated with the destruction of mitochondrial cristae in mice colorectal tumors. To further confirmed the role of OPA1 processing in regulating mitochondrial cristae in mice colorectal tumor, control, OPA1 knockout (OPA1 KO), OPA1 knockdown (shOPA1), or OMA1-Yme1L double knockout (OPA1 processing is completely inhibited) HCT116 cells were injected into nude mice. The transmission electron microscope (TEM) analysis revealed that control (OPA1 is processed due to hypoxic environment), OPA1 KO or shOPA1 HCT116 xenograft tumor cells maintained very little mitochondrial cristae (Fig EV3H-L of the revised manuscript, new data), but Yme1l-OMA1 double knockout HCT116 xenograft tumor cells, in which OPA1 processing or degradation is completely inhibited, displayed remarkable increased mitochondrial cristae (Fig EV3H-L of the revised manuscript, new data). These data suggest that OMA1 (and Yme1L)-mediated OPA1 processing in tumor directly contributes to mitochondrial cristae loss. In addition, we agree with the reviewer's opinion that some other factors may also regulate mitochondrial cristae during tumor development, because OPA1 is not the only factor regulating mitochondrial cristae biogenesis, and some other proteins such as MICOS and dimeric F1FO-ATP synthase also play important role in mitochondrial cristae formation (Cogliati et al, 2016). We have added and discussed these data in the revised manuscript.

References

An HJ, Cho G, Lee JO, Paik SG, Kim YS, Lee H (2013) Higd-1a interacts with Opa1 and is required for the morphological and functional integrity of mitochondria. *Proc Natl Acad Sci U S A* 110: 13014-13019

Anand R, Wai T, Baker MJ, Kladt N, Schauss AC, Rugarli E, Langer T (2014) The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. The Journal of Cell Biology 204: 919-929

Cogliati S, Enriquez JA, Scorrano L (2016) Mitochondrial Cristae: Where Beauty Meets Functionality. *Trends in biochemical sciences* 41: 261-273

DeVay, R.M., Dominguez-Ramirez, L., Lackner, L.L., Hoppins, S., Stahlberg, H., and Nunnari, J. (2009). Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. *The Journal of cell biology* 186, 793-803.

MacVicar T, Ohba Y, Nolte H, Mayer FC, Tatsuta T, Sprenger H-G, Lindner B, Zhao Y, Li J, Bruns C et al (2019) Lipid signalling drives proteolytic rewiring of mitochondria by YME1L. *Nature* 575: 361-365

Patten, D.A., Wong, J., Khacho, M., Soubannier, V., Mailloux, R.J., Pilon-Larose, K., MacLaurin, J.G., Park, D.S., McBride, H.M., Trinkle-Mulcahy, L., et al. (2014). OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. *The EMBO journal* 33, 2676-2691.

Song, Z., Ghochani, M., McCaffery, J.M., Frey, T.G., and Chan, D.C. (2009). Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. *Molecular biology of the cell* 20, 3525-3532.

The requirement of OMA1 for HIF1a stabilization is striking and the authors explain this by reduced ROS formation in the absence of OMA1. However, how OMA1 support ROS formation remains enigmatic.

Thanks for the reviewer's comments. It has been reported that the mitochondrial respiratory chain is the major source of ROS in eukaryotic cells, and the dysfunction of mitochondrial respiratory chain complexes is associated with mitochondrial ROS (mtROS) production (Dröse & Brandt, 2012; Kausar et al, 2018). In our manuscript, we identified that mitochondrial respiratory chain complexes components NDUFB5, NDUFB6, COX4L1 and NDUFA4 are proteolytic substrates of OMA1 (Fig 5 of the revised manuscript), while NDUFB5 and NDUFB6 are the subunits of NADH dehydrogenase (complex I), and COX4L1 and NDUFA4 are the components of the cytochrome c oxidase (complex IV). The blue native PAGE (BN-PAGE) assay revealed that knockdown (KD) of NDUFB5 or NDUFB6 remarkably reduced mitochondrial respiratory complex I in HCT116 cells (Fig EV4E of the revised manuscript, new data), and COX4L1 knockdown or NDUFA4 knockout (KO) markedly reduced mitochondrial respiratory complex IV (Fig EV4E of the revised manuscript, new data), indicating that OMA1-mediated degradation or processing of NDUFB5, NDUFB6, COX4L1 and NDUFA4 impairs mitochondrial respiratory chain under hypoxia. We then investigated the effect of NDUFB5, NDUFB6, COX4L1, or NDUFA4 depletion on mtROS production. The results indicated that mtROS was remarkably increased in NDUFB5 KD, NDUFB6 KD, COX4L1 KD or NDUFA4 KO HCT116 cells (Fig EV5A and B of the revised manuscript, new data). Therefore, under hypoxic conditions, OMA1 mediates NDUFB5, NDUFB6, COX4L1 and NDUFA4 processing or degradation to impair mitochondrial respiratory complexes, thereby increasing mtROS production. We have added the data and discussed the issue in the revised manuscript.

References

Dröse S, Brandt U (2012) Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Advances in experimental medicine and biology* 7**48**: 145-69

Kausar S, Wang F, Cui H (2018) The Role of Mitochondria in Reactive Oxygen Species Generation and Its Implications for Neurodegenerative Diseases. *Cells* **7:** 274

The identification of novel substrates should be more directly confirmed using chase experiments upon cycloheximide treatment or in hypoxic conditions in WT, OMA1 KO and OMA1 KO cells complemented with WT-OMA1/proteolytic inactive OMA1.

We appreciate the reviewer's suggestions. According to the reviewer's suggestions, WT, OMA1 KO, and OMA1 KO HCT116 cells expressing OMA1-Flag or OMA1-E324Q-Flag (proteolytic inactive OMA1) were cultured in hypoxia (1% O_2) for 0, 24, or 48 hours. Cell lysates were then analyzed by Western blotting. In response to hypoxia, the protein levels of NDUFB5, NDUFB6, COX4L1, and NDUFA4 were markedly decreased in WT or OMA1 KO cells expressing OMA1-Flag (Appendix Fig S3C of the revised manuscript, new data). However, OMA1 KO or OMA1 KO cells expressing E324Q-Flag markedly inhibited the reduction of NDUFB5, NDUFB6, COX4L1, and NDUFA4 (Appendix Fig S3C of the revised manuscript, new data). these data suggest that OMA1 proteolytic activity regulates the degradation of NDUFB5, NDUFB6, COX4L1, and NDUFA4. It should be noted that the protein levels of NDUFB5, NDUFB6, COX4L1, and NDUFA4 were still decreased at 24h or 48h of hypoxia, probably due to mitophagy or activated some other mitochondrial proteases under hypoxia.

In addition, as suggested by the reviewer, WT, OMA1 KO, and OMA1 KO HCT116 cells expressing OMA1-Flag or OMA1-E324Q-Flag (proteolytic inactive OMA1) were also treated with cycloheximide (CHX, an inhibitor of protein synthesis) plus CCCP (OMA1 activity stimulator) for 0, 2, 4, or 6 hours. Cell lysates were then analyzed by Western blotting. Upon CHX plus CCCP treatment, the protein levels of NDUFB5, NDUFB6, COX4L1, and NDUFA4 were quickly reduced in control cells or OMA1 KO cells expressing OMA1-Flag cells, but the reduction of these proteins was remarkably inhibited in OMA1 KO cells or OMA1 KO cells expressing E324Q-Flag OMA1 (Fig 5F of the revised manuscript, new data).

Overall, these data further suggest that NDUFB5, NDUFB6, COX4L1, and NDUFA4 are the novel substrates of mitochondrial protease OMA1.

Data quality is generally high but there are some points to be made. Representative blots are used throughout without quantification. The authors should at least indicate the number of experiments performed. White gaps in western blots of potential OMA1 substrates between WT and OMA1-KO lanes e.g EV6 B and 4F should be referred to, which could strongly affect the interpretation.

We appreciate the reviewer's comments and suggestions. According to the reviewer's

suggestions, we performed densitometry analysis to quantify the bands of Western blots in Figures 4A, 4B, 5G, 6A-C, EV3C and EV4E of the revised manuscript by Image software, and the data were provided and displayed in the revised manuscript. In addition, the number of experiments performed was indicated in the related figure legends of the revised manuscript.

Additionally, the white gaps between the two lanes of Western blots are due to other unrelated bands between the bands in the blots being cut off. As suggested by the reviewer, we repeated Western blotting analysis and replaced Figures 4F, EV5D and 6B in the previous manuscript with new figures (Fig 5F, EV3D and Appendix Fig S3B of the revised manuscript).

Except for panel G, the contribution of Fig.5 to the paper's principle message is unclear and unhelpful. Without quantification, molecular weight markers and SDS-PAGE loading controls, the BN-PAGEs in A and B cannot really be interpreted.

As suggested by the reviewer, we added the quantification, molecular weight markers and SDS-PAGE loading controls in the Figures 6A-C of the revised manuscript (Fig 5 of the previous manuscript).

Mitochondrial respiratory chain is the predominant source of ROS in eukaryotic cells, and dysfunction of mitochondrial respiratory chain complexes is associated with mitochondrial ROS (mtROS) production (Dröse & Brandt, 2012; Kausar et al, 2018). Moreover, mitochondrial respiratory chain plays an important role in generating energy by oxidative phosphorylation. But how cancer cells coordinate glycolysis and oxidative phosphorylation in hypoxia for energy metabolism remains largely unknown. Therefore, we investigated the role of OMA1 in the assembly of mitochondrial respiratory chain complexes under hypoxic conditions, which can help us to understand the underlying mechanism of OMA1 regulating mtROS production and oxidative phosphorylation in hypoxia.

References

Dröse S, Brandt U (2012) Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Advances in experimental medicine and biology* 7**48**: 145-69

Kausar S, Wang F, Cui H (2018) The Role of Mitochondria in Reactive Oxygen Species Generation and Its Implications for Neurodegenerative Diseases. *Cells* **7:** 274

Bioinformatic analysis reveals that OMA1 mRNA expression in enhanced in human tumours but this does not appear to be the mechanism that regulates its activity in hypoxic cells (Fig. EV2), perhaps the authors can speculate on how OMA1 is stimulated during hypoxia.

We agree the reviewer's opinion that the upregulation of OMA1 mRNA expression in human tumours does not appear to be the mechanism that regulates its activity in hypoxic cells. We also observed that hypoxia (24h) does not induce the upregulation of OMA1 mRNA in HCT116 cells *in vitro* (Appendix Fig S1 of the revised manuscript). However, hypoxia can induce mitochondrial damage and oxidative stress, which may active OMA1. In the revised manuscript, OMA1 protein activity is increased by hypoxia due to self-cleavage or autocatalytic turnover (Fig 1D-G of the revised manuscript), consistent with previous reports that OMA1 is activated by CCCP treatment by self-cleavage and auto-degradation (Baker et al, 2014; Zhang et al, 2014). In addition, hypoxia can lead to the reduction of mitochondrial membrane potential (Solaini et al, 2010), which also contributes to the activation of OMA1. We have discussed the issue in the revised manuscript.

References

Baker MJ, Lampe PA, Stojanovski D, Korwitz A, Anand R, Tatsuta T, Langer T (2014) Stress-induced OMA1 activation and autocatalytic turnover regulate OPA1-dependent mitochondrial dynamics. *The EMBO journal* 33: 578-593

Zhang K, Li H, Song Z (2014) Membrane depolarization activates the mitochondrial protease OMA1 by stimulating self-cleavage. *EMBO reports* 15: 576-585

Solaini G, Baracca A, Lenaz G, Sgarbi G (2010) Hypoxia and mitochondrial oxidative metabolism. *Biochimica et biophysica acta* 1797: 1171-1177

The authors do not provide evidence for autocatalysis of OMA1 in the tumour situation or hypoxia, which they describe as a potential "stop button" in hypoxic adaptation. Previous work has shown that OMA1 is degraded by YME1L in hypoxia (MacVicar et al., 2019 Nature). To conclusively test OMA1 autocatalysis in colon cancer cells, the authors could see whether proteolytic-dead OMA1 is degraded upon hypoxia in OMA1 KO cells.

As suggested by the reviewer, we cultured WT, OMA1 KO, and OMA1 KO HCT116 cells expressing OMA1-Flag or OMA1-E324Q-Flag (proteolytic inactive OMA1) in hypoxia (1% O2) for 0, 24, or 48 hours. Cell lysates were then analyzed by Western blotting with antibody against OMA1. We observed that the protein levels of OMA1 (WT cells) or OMA1-Flag (OMA1 KO cells expressing OMA1-Flag) were greatly decreased in hypoxia (48 hours) compare to normoxia (0 hour) due to cleavage and degradation (Fig 1F and G of the revised

manuscript, new data), but the reduction of OMA1-E324Q-Flag (OMA1 KO cells expressing OMA1-E324Q-Flag) was inhibited in hypoxia (48 hours) (Fig 1F and G of the revised manuscript, new data), indicating that OMA1 undergoes auto-proteolytic cleavage and auto-degradation, consisting with the previous reports (Baker et al, 2014; Zhang et al, 2014). Importantly, OMA1-E324Q-Flag was still mildly reduced in hypoxia (48 hours) (Fig 1F and G of the revised manuscript, new data), which indicates that OMA1 can be degraded or cleaved by some other proteases. Our findings are consistent with the recent report that OMA1 is degraded by Yme1L in hypoxia (MacVicar et al, 2019). Therefore, OMA1 cooperates with Yme1L to degrade OMA1 in hypoxia. We have added the data and discussed the issue in the revised manuscript.

References

Baker MJ, Lampe PA, Stojanovski D, Korwitz A, Anand R, Tatsuta T, Langer T (2014) Stress-induced OMA1 activation and autocatalytic turnover regulate OPA1-dependent mitochondrial dynamics. *The EMBO journal* 33: 578-593

MacVicar T, Ohba Y, Nolte H, Mayer FC, Tatsuta T, Sprenger HG, Lindner B, Zhao Y, Li J, Bruns C *et al* (2019) Lipid signalling drives proteolytic rewiring of mitochondria by YME1L. *Nature* 575: 361-365

Zhang K, Li H, Song Z (2014) Membrane depolarization activates the mitochondrial protease OMA1 by stimulating self-cleavage. *EMBO reports* 15: 576-585

Method/reference for the HCT116 OMA1 KO cell line is missing

Thanks to the reviewer for the suggestions. We have described the method of HCT116 OMA1 KO cell line in the section of "Generation of knockout cells" of "Materials and Methods" of the revised manuscript.

Referee #2:

In this study, Wu and colleagues investigate the role of OMA1 protease in metabolic rewiring and its relationship in cancer. Starting from showing a correlation between OMA1 expression and colorectal tumour development they progress to show that loss of OMA1 inhibits tumourigenesis in two models (AOM/DSS, xenograft). They then show that OMA1 promotes HIFalpha expression underhypoxia, linking to the effects of glycolysis described earlier. Investigating the mechanistic basis for this, they describe that under conditions of hypoxia or CCCP uncoupler, OMA1 cleaves various respiratory chain complex proteins, affecting complex levels, providing a means of metabolic rewiring towards a glycolytic phenotype. The study is well-performed, dataset extensive and largely supports the authors' conclusions. I have a few points outstanding that should be addressed.

- In my opinion, based on the authors data, how OMA1 promotes a shift to glycolysis, may be multifaceted - potentially by HIF1 alpha upregulation but equally via degradation of respiratory complex proteins - the importance of the latter is difficult to define (given that many substrates are involved), however the importance of HIF1 alpha to OMA1 mediated rewiring (i.e. shift to glycolysis) should be readily testable by inhibiting HIF1 alpha expression.

We sincerely thank the reviewer for the comments and suggestions. According to the reviewer's suggestions, we performed HIF-1α knockdown in WT and OMA1 KO HCT116 cells, and then detected glycolytic metabolism in cells cultured under normoxia and hypoxia for 12 hours. Western blot analysis revealed that HIF-1α was successfully depleted after HIF-1α knockdown in WT and OMA1 KO HCT116 cells under hypoxia (Fig EV2A of the revised manuscript, new data). Compare with control cells, OMA1 KO remarkably decreased glucose uptake and lactate production under hypoxia (Fig EV2B and C of the revised manuscript, new data). However, HIF-1α knockdown significantly inhibited glucose uptake and lactate production both in control and OMA1 KO under hypoxia (Fig EV2B and C of the revised manuscript, new data). These data indicate that OMA1 regulates glycolysis in a HIF-1α dependent manner.

In addition, we agree with the reviewer's opinion that the many respiratory complex proteins are degraded under hypoxia. In our manuscript, we focus on the degradation of OMA1 substrates under hypoxia. To further investigate the role of OMA1-mediated degradation of respiratory complexes components under hypoxia in the shift to glycolysis, we detected the glycolytic metabolism in control, sh*NDUFB5* (NDUFB5 KD), sh*NDUFB6* (NDUFB6 KD), sh*COX4L1* (COX4L1 KD), or NDUFA4 KO cells. Notably, glucose uptake and lactate

production were significantly increased in shNDUFB5, shNDUFB6, shCOX4L1, or NDUFA4 KO cells compared with control cells (Fig EV5C and D of the revised manuscript, new data). Taken together, these data suggest that OMA1 promotes a shift to glycolysis by upregulating HIF-1α and degrading respiratory complexes components.

- Extending on this point, the authors state (Fig 3) that ROS generated by OMA1 is required for HIF1 alpha upregulation - this is not directly tested (i.e. through use of ROS scavenger), either the claim should be moderated somewhat, or an expt. to test this should be performed.

As suggested by the reviewer, we treated WT and OMA1 KO HCT116 cells with DMSO, Rotenone (ROS inducer) or NAC (ROS scavenger) in normoxia or hypoxia for 12 hours, and then analyzed HIF-1α stability, glucose uptake and lactate production. Consistently, OMA1 KO inhibited the upregulation of HIF-1α and glycolytic metabolism under hypoxia (Fig 4G-I of the revised manuscript, new data). In addition, compared with DMSO treatment, Rotenone treatment remarkably increased the protein levels of HIF-1α in control and OMA1 KO cells in hypoxia, while NAC treatment inhibited hypoxia-induced upregulation of HIF-1α both in control and OMA1 KO cells (Fig 4G of the revised manuscript, new data). These data suggesting that OMA1 KO inhibits hypoxia-induced upregulation of HIF-1α by regulating ROS. Moreover, Rotenone treatment promoted lactate production and glucose uptake in OMA1 KO cells under hypoxia, but NAC treatment decreased lactate production and glucose uptake both in control and OMA1 KO cells (Fig 4H and I of the revised manuscript, new data). These results demonstrate OMA1-mediated ROS production is required for HIF-1α upregulation in hypoxia.

- The error bars on some of the metabolic assays (e.g. Fig 2) appear impossibly small to represent biological replicates, are they instead technical replicates?

We previously used the samples with the same number of cells (at least 3 samples for each cell lines) and performed metabolic assays at the same time, which resulted in the error bars on some of the metabolic assays were very small. To solve this problem, we further repeated some metabolic assays at different time, and provided new data (Fig 3 of the revised manuscript) in the revised manuscript.

- The ms. is data rich, I do realise journal figure limits etc. but ideally the intial results section should be presented in figure 1 (not various extended figures). The grammar, flow of the text can also be improved to help the reader.

We appreciate the reviewer's comments and suggestions. According to the reviewer's suggestions, we put Figure EV1 to be Figure 1. In addition, we have tried our best to improve the grammar of the revised manuscript.

Referee #3:

The work by Wu et al describes the involvement of OMA1 in the progression and generation of colorectal cancer. The data shown are interesting and improve our understanding of the mechanisms linking hypoxia and metabolic reprogramming within the tumor microenvironment. However, some points need to be addressed before accepting the main conclusions of the paper:

Major points:

The authors report that OMA1 is activated under hypoxia, while its total levels do not change. This may indicate that OMA1 is activated post-translationally. Several works (Simula et al., 2020 - Jiang et al., 2014 PNAS and many others) indicate that active OMA1 can be detected as a shorter fragment compared to full-length protein. Authors should check.

We appreciate the reviewer's comments and suggestions. OMA1 is activated (cleaved) to produce shorter fragment of OMA1 (S-OMA1) under certain stresses (Baker et al., 2014; Jiang et al, 2014; Simula et al, 2020; Zhang et al., 2014). As suggested by the reviewer, we cultured WT, OMA1 KO, and OMA1 KO HCT116 cells expressing OMA1-Flag or OMA1-E324Q-Flag (proteolytic inactive OMA1) in hypoxia (1% O_2) for 0, 24, or 48 hours. The cell lysates were then assessed by Western blotting with antibody against OMA1. The short form of OMA1 (S-OMA1) in OMA1 KO HCT116 cells expressing OMA1-Flag, were detected in hypoxia (Fig 1F of the revised manuscript, new data). However, S-OMA1 was not detected in OMA1 KO HCT116 cells expressing OMA1-E324Q-Flag in hypoxia (Fig 1F of the revised manuscript, new data), because the protease activity of OMA1 is required for the generation of S-OMA1. In addition, we didn't detect the S-OMA1 in WT cells under hypoxia, probably due to the low stability of S-OMA1 or the low quality of antibody. We have added the data and the related references in the revised manuscript.

References

Baker MJ, Lampe PA, Stojanovski D, Korwitz A, Anand R, Tatsuta T, Langer T (2014) Stress-induced OMA1 activation and autocatalytic turnover regulate OPA1-dependent mitochondrial dynamics. *The EMBO journal* 33: 578-593

Jiang X, Jiang H, Shen Z, Wang X (2014) Activation of mitochondrial protease OMA1 by Bax and Bak promotes cytochrome c release during apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 111: 14782-14787

Soriano ME *et al* (2020) JNK1 and ERK1/2 modulate lymphocyte homeostasis via BIM and DRP1 upon AICD induction. *Cell death and differentiation.* doi: 10.1038/s41418-020-0540-1

Zhang K, Li H, Song Z (2014) Membrane depolarization activates the mitochondrial protease OMA1 by stimulating self-cleavage. *EMBO reports* 15: 576-585

Data in Figures 2A-D are interpreted using student t-test. However, since there are more than 2 samples in each experimental layout, an ANOVA (two-ways) should be performed instead (also because the differences are very little and must be properly statistically tested).

We appreciate the reviewer's thoughtful suggestions. As suggested, we have performed a two-way ANOVA in Figures 3A-D (Figures 2A-D of the previous manuscript) of the revised manuscript.

Western blot images in Figures 3A, 3B, 4F, 5A-B and EV5C should be accompanied by a graph indicating the quantification of the bands. In addition, image 5B seems to show that OMA1-KO is not able to restore complexes I-IV under hypoxia compared to control hypoxia conditions, while the authors state the opposite. Can the Authors clarify please? The image does not support author conclusions. Different representative images should be chosen and adding quantification may help. In figure EV5C the rescue effect on Opa1 due to OMA1-KO is not evident.

We thank the reviewer for his/her comments and suggestions. According to the reviewer's suggestion, we have added the quantitative graphs of Western blot images in Figures 3A, 3B, 4F, 5A-B and EV5C (4A, 4B, 5G, 6A-B and EV3C of the revised manuscript). And we regret for the clerical error that OMA1 KO can restore complexes I-IV under hypoxia compared with control cell in hypoxia. In fact, OMA1 KO can only restore complexes I and IV in cells exposed to hypoxia. And we have repeated and replaced them with more representative graphs, and we also provided the data of quantification (Fig 6B of the revised manuscript). In addition, we performed blue native PAGE (BN-PAGE) to analyze the integrity of mitochondrial respiratory chain complexes in control, sh*NDUFB5* (NDUFB5 knockdown), sh*NDUFB6* (NDUFB6 knockdown), sh*COX4L1* (COX4L1 knockdown), and NDUFA4 KO cells. Notably, compared with control cells, sh*NDUFB5* or sh*NDUFB6* cells showed a significant decrease of mitochondrial respiratory chain complex I, and sh*COX4L1* and NDUFA4 KO cells displayed a significant decrease of mitochondrial respiratory chain complex IV (Fig EV4E of the revised manuscript, new data). There results indicate that NDUFB5 and NUDFB6 are required for the integrity (or assembly) of mitochondrial respiratory chain complex I, and COX4L1 and NURFA4 are required for the integrity (or assembly) of mitochondrial respiratory chain complex IV. Furthermore, OMA1 KO restored the degradation of NDUFB5, NDUFB6, COX4L1, and NDUFA4 under hypoxic conditions (Fig 5G of the revised manuscript). Taken

together, OMA1 KO restored complexes I and IV under hypoxia probably due to the rescue of these substrates.

Additionally, we quantified the bands of OPA1 and L-OPA1 in Figure EV3C (EV5C of the previous manuscript) of the revised manuscript. The result revealed that the level of OPA1, especially L-OPA1 in OMA1 KO colorectal tumors was significantly higher than that in control colorectal tumors (Fig EV3C of the revised manuscript), suggesting that OMA1 KO has a rescue effect on OPA1 in colorectal tumors.

Authors state that OMA1 regulates HIF1a (and then glycolysis) through ROS production. However, a rescue experiment showing that restoration of ROS in OMA1-KO cells does increase HIF1 (and glycolysis) is missing. As shown, the mechanism lacks an important step to be firmly established.

Thanks to the reviewer for the suggestions. According to the reviewer's suggestions, we treated WT and OMA1 KO HCT116 cells with DMSO, Rotenone (to restore ROS) or NAC (ROS scavenger) in normoxia or hypoxia for hours, and then analyzed HIF-1α stability, glucose uptake and lactate production. Consistently, OMA1 KO inhibited the upregulation of HIF-1α and glycolysis under hypoxia (Fig 4G-I of the revised manuscript, new data). In addition, compared with DMSO treatment, Rotenone treatment remarkably increased the protein levels of HIF-1α both in control and OMA1 KO cells in hypoxia, while NAC treatment inhibited hypoxia-induced upregulation of HIF-1α in control and OMA1 KO cells (Fig 4G of the revised manuscript, new data). These data suggesting that OMA1 KO inhibits hypoxia-induced upregulation of HIF-1α by regulating ROS. Moreover, Rotenone treatment promoted lactate production and glucose uptake in OMA1 KO cells under hypoxia, but NAC treatment decreased glycolysis both in control and OMA1 KO cells (Fig 4H and I of the revised manuscript, new data). These results demonstrate OMA1- regulates HIF-1α stability and subsequent glycolysis in hypoxia by modulating ROS production.

Minor Points:

It is not clear what the asterisks indicate in Fig 1B. How was the statistics calculated? ANOVA TW should be performed.

Thanks for the reviewer's suggestions. The asterisks in Fig 1B of previous manuscript (Fig 2B of the revised manuscript) indicate the statistical significance of body weight changes at

different days and changes between WT and OMA1 KO mice during AOM/DSS treatment. The statistical significance was previously assessed by a two-tailed Student's t-test. As suggested by the reviewer, we used a two-way ANOVA to evaluate the statistical significance in Fig 2B of the revised manuscript.

A western blot showing the over-expression of Flag-OMA1 related to Figure 2 should be shown at least in supplementary.

As suggested by the reviewer, we added the Western blot showing the over-expression of OMA1-Flag related to Figure 2 of the previous manuscript (Figure 3 of the revised manuscript) in Fig 3A of the revised manuscript. In brief, WT, OMA1 KO and OMA1 KO HCT116 cells expressing Flag-OMA1 were lysed and analyzed by Western blotting with antibody against OMA1 (Fig 3A of the revised manuscript, new data).

The experimental setup to induce hypoxia in vitro should be better explained in the Methods section.

As suggested by the reviewer, the experimental setup to induce hypoxia in vitro has been added to the section of "Cell Culture Reagents and Antibodies" in "Materials and Methods" of the revised manuscript, as shown in bellow:

For hypoxia experiments, cells were cultured in a sealed incubator chamber with a ProOX C21 O_2/CO_2 controller (BioSpherix, USA). The hypoxic conditions consisted of 1% O_2, 5% CO_2, and 94% N_2. The oxygen concentration was kept at 1% by continuous infusion of N_2.

1st Revision - Editorial Decision 16th Oct 2020

Dear Dr. Song,

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees.

All referees acknowledge that the revised manuscript significantly improved. Whereas referees #2 and 3 support publication, referee #1 has outstanding concerns, mainly regarding the proposed mechanism whereby OMA1 coordinates glycolysis and mitochondrial respiration during colorectal cancer progression. I have discussed these points further with the referees #2 and 3. Please activate 'track changes on' while performing the below alterations.

- To address the concern that starts as "It remains a major concern that the authors describe rather subtle metabolic deficiencies in OMA1 KO cells in vitro..." please tone down your conclusions, stating that OMA1 loss may be contributing to enhanced tumorigenesis through the metabolic mechanism described herein, though additional OMA1 effects may also play key roles.

- To address the concern "Similarly, the authors show that OPA1 KO cells are more vulnerable to glucose deprivation.." please tone down your conclusions by stating that the effects may be via OMA1 regulation of OPA1 as this is not directly demonstrated as it stands. Also, please acknowledge the variability you observe in your HIF1alpha results.

- Please address the concern "The statistical evaluation of the data is another major concern, considering the in part subtle effects..." by clarifying the nature of the replicates, what error bars represent, which type of statistical analysis used and so on.

- Please address the concern "The identification of OMA1 substrates still remains controversial..." by toning down the mentioned claims and acknowledging the controversy in the field as stated by referee #1. Moreover, please tone down the claims regarding self degradation of OMA1.

In addition, please address these editorial points:

• I made some minor changes in the items below. Please take a look and confirm, or feel free to suggest further changes.
o In line with the referee reports, I recommend the following title: "OMA1 reprograms metabolism under hypoxia to promote colorectal cancer development"
o Similarly, I made changes in the Abstract: Many cancer cells maintain enhanced aerobic glycolysis due to irreversible defective mitochondrial oxidative phosphorylation (OXPHOS). This phenomenon, known as the Warburg effect, is recently challenged because most cancer cells maintain OXPHOS. However, how cancer cells coordinate glycolysis and OXPHOS remains largely unknown. Here, we demonstrate that OMA1, a stress-activated mitochondrial protease, promotes colorectal cancer development by driving metabolic reprogramming. OMA1 knockout suppresses colorectal cancer development in AOM/DSS and xenograft mice models of colorectal cancer. OMA1-OPA1 axis is activated by hypoxia, increasing mitochondrial ROS to stabilize HIF-1α, thereby promoting glycolysis in colorectal cancer cells. On the other hand, under hypoxia, OMA1 depletion promotes accumulation of NDUFB5, NDUFB6, NDUFA4 and COX4L1, supporting that OMA1 suppresses OXPHOS in colorectal cancer. Therefore, our findings support a role for OMA1 in coordination of glycolysis and OXPHOS to promote colorectal cancer development, and highlight OMA1 as a potential target for colorectal cancer therapy.

• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• Please add an 'Author Contributions' section.
• We noticed that Figure 4I was not called out in the text whereas Figure 6D was only called out in Discussion.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have carefully considered my points when revising the manuscript and also added new experiments. Although I appreciate the efforts of the authors, I am not convinced that the provided evidence supports the proposed role of OMA1 in coordinating glycolysis and oxidative phosphorylation. It remains a major concern that the authors describe rather subtle metabolic deficiencies in OMA1 KO cells in vitro (e.g. Fig. 3, EV2) to explain the convincingly documented effects of OMA1 loss in the AOM/DSS model. The authors now added experiments showing that the loss of putative OMA1 substrates, core subunits of the respiratory chain, affect glucose uptake, glycolysis and lactate production. This is expected (and well-documented) for respiratory deficient cells and does not allow to conclude that the subtle changes in respiratory complex levels observed in OMA1 KO (Fig. 6A/B) have similar effects. Similarly, the authors show that OPA1 KO cells are more vulnerable to glucose deprivation (Fig. 3-H, EV1D/E). This is expected for respiratory deficient cells and does not allow to conclude that OMA1 promotes glycolysis by regulating OPA1. Another example is the conclusion of the authors that OMA1-regulated glycolysis depends on HIF1a. The authors provide some evidence that loss of OMA1 decreased glucose uptake and lactate production in hypoxia (Fig. 3). To examine HIF1a dependence, they depleted HIF1a and show that this also inhibited glucose uptake and lactate production (as expected) (Fig. EV2A-C). However, this

does not allow to conclude that the effect in OMA1 KO cells depends on HIF1a. These are only examples of a number of apparent over-statements in the manuscript.

The statistical evaluation of the data is another major concern, considering the in part subtle effects. The error bars appear extremely small for metabolomic experiments and for quantifications of immunoblots (SDS-PAGE and BN-PAGE). It remains unclear in the manuscript and the rebuttal letter, if technical or biological replicates are shown, which makes it impossible to assess the significance of some of the small effects.

The requirement of OMA1 for HIF1a stabilization is intriguing and the authors have added new experiments (Fig. 4G) to link this observation to ROS formation. However, the new Fig. 4G and Fig. EV2 hardly show any stabilizing effect of OMA1 loss on HIF1a levels. This apparent variability in HIF1a levels in OMA1 KO cells is difficult to reconcile with the quantification in Fig 4A.

The identification of OMA1 substrates still remains controversial. Although the new CHX pulse chase experiments are interesting and support degradation of some respiratory chain subunits by OMA1, the experiment in Appendix Fig S3C does not show any or only very subtle differences in the stability of those proteins under hypoxia in WT, OMA1 KO or OMA1 KO cells expressing WT or E324Q OMA1. Related to this point, the authors examined autocatalytic turnover of OMA1 monitoring the stability of over-expressed proteolytically inactive OMA1 in Fig. 1F/G, but observe only a very minor difference, which in my opinion does not allow to conclude autocatalytic turnover.

Referee #2:

The authors have comprehensively addressed all my comments

Referee #3:

In this revised version of the manuscript the Authors fulfilled adequately all the referee's concerns, thus the manuscript might be now suitable for publication on EMBO Reports.

Referee #1:

The authors have carefully considered my points when revising the manuscript and also added new experiments. Although I appreciate the efforts of the authors, I am not convinced that the provided evidence supports the proposed role of OMA1 in coordinating glycolysis and oxidative phosphorylation. It remains a major concern that the authors describe rather subtle metabolic deficiencies in OMA1 KO cells in vitro (e.g. Fig. 3, EV2) to explain the convincingly documented effects of OMA1 loss in the AOM/DSS model. The authors now added experiments showing that the loss of putative OMA1 substrates, core subunits of the respiratory chain, affect glucose uptake, glycolysis and lactate production. This is expected (and well-documented) for respiratory deficient cells and does not allow to conclude that the subtle changes in respiratory complex levels observed in OMA1 KO (Fig. 6A/B) have similar effects. Similarly, the authors show that OPA1 KO cells are more vulnerable to glucose deprivation (Fig. 3-H, EV1D/E). This is expected for respiratory deficient cells and does not allow to conclude that OMA1 promotes glycolysis by regulating OPA1. Another example is the conclusion of the authors that OMA1-regulated glycolysis depends on HIF1a. The authors provide some evidence that loss of OMA1 decreased glucose uptake and lactate production in hypoxia (Fig. 3). To examine HIF1a dependence, they depleted HIF1a and show that this also inhibited glucose uptake and lactate production (as expected) (Fig. EV2A-C). However, this does not allow to conclude that the effect in OMA1 KO cells depends on HIF1a. These are only examples of a number of apparent over-statements in the manuscript.

We appreciate the reviewer's comments and constructive criticisms. According to the reviewer's and editor's comments, we toned down the conclusions related to OMA1, OPA1 and HIF1a in the revised manuscript. For example, the statements "the effects may be via OMA1 regulation of OPA1", "OMA1 loss may be contributing to enhanced tumorigenesis through the metabolic mechanism described herein, though additional OMA1 effects may also play key roles", "OMA1 promotes glycolysis probably by stabilizing HIF-1α protein under hypoxia", and "OMA1 may regulate glycolysis in a HIF-1α dependent manner", have been provided in the revised manuscript.

The statistical evaluation of the data is another major concern, considering the in part subtle effects. The error bars appear extremely small for metabolomic experiments and for quantifications of immunoblots (SDS-PAGE and BN-PAGE). It remains unclear in the manuscript and the rebuttal letter, if technical or biological replicates are shown, which makes it impossible to assess the significance of some of the small effects.

Thanks for the reviewer's comments and constructive criticisms. Some error bars appear small for

metabolic experiments and for quantifications of immunoblots (SDS-PAGE and BN-PAGE), because some date were further analyzed and converted to be the related data (the ratio of control data), causing some error bar to be small. In addition, some data themselves are small (such as WT tumors data in Figure 6C) compared with control, thereby leading to small error bars. Additionally, we just showed a positive (+) error bar in the figures of the previous version of manuscript, which looks small, so we showed ± error bar in the revised manuscript. Moreover, we performed experiments more than 3 times, according to the reviewer's comments, certain small error bars are improved with the new data.

The requirement of OMA1 for HIF1a stabilization is intriguing and the authors have added new experiments (Fig. 4G) to link this observation to ROS formation. However, the new Fig. 4G and Fig. EV2 hardly show any stabilizing effect of OMA1 loss on HIF1a levels. This apparent variability in HIF1a levels in OMA1 KO cells is difficult to reconcile with the quantification in Fig 4A.

According to the reviewer's comments, we performed the experiment related to Fig EV2A. The new Fig EV2A is provided in the revised manuscript.

The identification of OMA1 substrates still remains controversial. Although the new CHX pulse chase experiments are interesting and support degradation of some respiratory chain subunits by OMA1, the experiment in Appendix Fig S3C does not show any or only very subtle differences in the stability of those proteins under hypoxia in WT, OMA1 KO or OMA1 KO cells expressing WT or E324Q OMA1. Related to this point, the authors examined autocatalytic turnover of OMA1 monitoring the stability of over-expressed proteolytically inactive OMA1 in Fig. 1F/G, but observe only a very minor difference, which in my opinion does not allow to conclude autocatalytic turnover.

We appreciate the reviewer's comments and constructive criticisms. It should be noted that some mitochondrial proteins including OPA1 and mitochondrial respiratory chain subunits etc., probably are degraded or processed by multiple mitochondrial proteases including OMA1, Yme1L, or LONP1 etc. For example, OPA1 can be processed by both OMA1 and Yme1L. Therefore, t Appendix Fig S3C does not show very subtle differences in the stability of mitochondrial respiratory chain subunits under hypoxia in WT, OMA1 KO or OMA1 KO cells expressing WT or E324Q OMA1, indicating these proteins may also be degraded by other mitochondrial proteases. In addition, minor difference of OMA1 autocatalytic turnover between OMA1 KO cells expressing WT OMA1 and OMA1 KO cells expressing E324Q OMA1 was observed probably due to the

degradation of OMA1 by Yme1L in hypoxia. Thus, we toned down the claims about OMA1 self-degradation, and have stated that "OMA1-E324Q was still mildly reduced in hypoxia (48 hours) (Fig 1F and G), suggesting that OMA1 can be degraded or cleaved by some other proteases in hypoxia. Our findings are consistent with the recent report that OMA1 is degraded by Yme1L in hypoxia (MacVicar et al., 2019). Therefore, OMA1 may cooperate with Yme1L to degrade OMA1 in hypoxia" in our revised manuscript.

2nd Revision - Editorial Decision 3rd Nov 2020

Dear Prof. Song,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

I noted that the labels of the synopsis image is too small to read, especially the ones drawn in the mitochondria. Please increase the font size of the labels and send me the image per email, after which we can transfer the manuscript to our production team.

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Corresponding Author Name: Zhiyin Song
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-50827V2

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 iustified
- 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 the data were repeated at least three times. We have used at least triplicates in experiments in which statistics are shown.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	6-8week old male nude mice (n=5 per group) and 12-14 week old male C57BL/6 mice (n=11-12 per group) were used
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded once the study was initiated. All our criteria were pre-established.
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.	No steps were taken to minimize the effects of subjective bias, however, all samples were measured in biological triplicates to minimize experimental biases.
For animal studies, include a statement about randomization even if no randomization was used.	All the mice were chosen randomly.
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	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical tests were used appropraitely.
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, standard deviations (s.d.) or standard errors (s.e.m.) are shown where indicated.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	All the antibodies and reagents are referenced appropriately.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines used in this study are listed appropriatly and tested 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.	6-8 week old male nude mice obtained from Animal Research Center of Wuhan University were used in this study. C57BL/6J-Oma1-eKO1 mice were obtained from Shanghai Model Organisms Center, Inc. Mice were raised under SPF conditions (no Helicobacter pylori).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Studies on animals were conducted with approval from the Animal Care Committees of Wuhan University College of Life Sciences.
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.	Yes

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.	NA