EMBO reports

LINC00493-encoded microprotein SMIM26 exerts antimetastatic activity in renal cell carcinoma

Kun Meng, Shaohua Lu, Yu-ying Li, Li-ling Hu, Jing Zhang, Yun Cao, Yang Wang, Chris Zhiyi Zhang, and Qing-Yu He
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1st Editorial Decision **8th Nov 2022**

Dear Dr. Zhang,

Thank you for the transfer of your research manuscript to EMBO reports. I have now received the 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, the referees think that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here.

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 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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:

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3) 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

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

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 now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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, 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 for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Although the LINC00493-encoded SMIM26 has been identified by the authors and two other labs, its functions in cells remain unclear. In this manuscript, the authors functionally characterizes the microprotein and establishes its antimetastatic role in ccRCC. They showed that SMIM26 is a mitochondrial outer membrane protein, which is downregulated in clear cell renal cell carcinoma (ccRCC) and correlated with poor overall survival. Knockdown of LINC00493-binding protein PABPC4 reduced the expression of SMIM26. They further demonstrated that SMIM26, but not LINC00493, suppresses ccRCC growth and metastatic lung colonization.

They showed that SMIM26 interacted with acylglycerol kinase (AGK) and glutathione transport regulator SLC25A11 through its N-terminal. SMIM26-expressing cells had increased mitoGSH level, basal and maximal mitochondrial respiration rate, which could be markedly inhibited by SCL25A11 siRNAs. Moreover, SMIM26 suppresses the phosphorylation of AKT at Ser473 in ccRCC cells. Overexpression of SMIM26 increased the level of mitochondrial AGK. The authors suggested that SMIM26 retains AGK in mitochondria to inhibit its capability of catalyzing AKT phosphorylation, and consequently restricts ccRCC metastasis. Interestingly, SMIM26 promoted the interaction of AGK and SLC25A11.

This manuscript contains a lot of interesting data and their experiments are technically sound. However, in several places, I am afraid their interpretation/conclusions lack enough evidence. The underlying mechanism of anti-metastatic activity of SMIM26 in ccRCC needs develop further.

Major points:
1. The authors showed that N-terminal of SMIM26 interacted with both AGK and SLC25A11. They suggested that SMIM26 forms a protein complex with AGK and SLC25A11 in mitochondria and promotes the interaction between AGK and SLC25A11. Since both AGK and SLC25A11 binds the same N-terminal of SMIM26, they might associate with SMIM26 in a competitive manner. How does SMIM26 promote the AGK and SLC25A11 interaction deserve to study further. To check whether SMIM26 can form dimer or oligomer, or map the minimal motifs in N-terminal domain of SMIM26 required to interact with AGK and SLC25A11 might be helpful to understand this point.

2. The authors suggest that RNA binding protein PABPC4 regulates SMIM26 expression. Although the levels of PABPC4 in ccRCC are lower than the adjacent normal tissues, however, the authors did not show that PABPC4 expression is lower in ACSN than that in HEK293 cells. In Fig.3C, the authors have examined the levels of PABPC4 in these two cell lines individually, they need run the samples in the same gel to compare the levels of PABPC4 in these cell lines directly.

3. The authors claimed that "Blockage on the interaction of SMIM26-SCL25A11 by siRNAs dramatically abrogated the SMIM26-mediated suppression of cell migration and invasion (Fig. 5P and Fig. S6G)". Based on the method they used (siRNA to knockdown SLC25A11), the authors did not provide direct evidence to support that SMIM26 plays a regulative role in mitochondrial function and cell migration by binding to SLC25A11. Rescue experiment with full length and mutant SCL25A11 not binding with SMIM26 might be helpful.

4. Based on the proposed model that SMIM26 retains AGK in mitochondria to inhibit its capability of catalyzing AKT phosphorylation, while overexpression of AGK in SMIM26-expression cells rescued the AKT phosphorylation, the authors are recommended to examine whether the levels of AGK in cytoplasm (outside of mitochondria) and PTEN phosphorylation are increased when AGK is overexpressed in SMIM26-expression ccRCC cells.

Minor points:
1, In Fig.1F, The quantification result was not provided.
2, In Fig. 1H & 1J, Fig. 3C & 3E & 3G, etc, The western blots lack molecular weight labels.
3, SMIM26-mut-flag (start codon ATG was mutated to GGA and stop codon TAA was mutated to GCT) plasmids. Why the stop codon in the SMIM26-mut-Flag is also mutated? Is it necessary?

4, In Fig. 4E, how is the intensity of F-actin fluorescence quantified? The method part did not give a detailed description.
5, In Fig. 5I, The signals of immunoprecipitated Mut1 & Mut2 are much lower than the Full-length SMIM26. A better representative image is required.

Referee #2:

Declaration: I was involved in a previous evaluation process of this manuscript for another journal and provide my updated suggestions herein.

In the current manuscript Kun Meng and colleagues describe the expression and function of a small microprotein called SMIM26, which is encoded by the putative non-coding RNA LINC00493. The authors convincingly show that SMIM26 is expressed in a panel of human (cancer) cell lines. SMIM26 localizes to mitochondria and probably is inserted in the mitochondrial membrane. Using a custom-made anti-SMIM26 antibody that authors investigated the expression of this microprotein in a panel of normal and cancerous human tissues. They could detect a significant downregulation of SMIM26 protein, but not LINC00493 expression in kidney cancer (ccRCC). Since the expression of the SMIM26 microprotein and its host RNA showed a poor correlation in cells and tissues, the authors tried to identify post-transcriptional regulators of SMIM26. RNA pulldown and RIP assays revealed an interaction between LINC00493 and PABPC4 and knockdown of the latter reduced SMIM26 protein levels. Further cellular and molecular assays establish SMIM26 as an inhibitor of kidney cancer cell migration, invasion and metastasis potentially via its interaction with SLC2511 and AGK. These interactions enhance GSH import into mitochondria, decrease the fraction of AGK in the cytosol and thereby reduce AKT phosphorylation.

In summary, the authors performed a comprehensive panel of in vitro and in vivo experiments and could establish a critical function for a novel microprotein regulating cancer cell motility in a human cancer entity. Therefore, this study is relevant and important as it sheds light into a new and understudied fraction of the human proteome encoded by recently suggested non-coding transcripts.
However, before this manuscript can be accepted for publication, the authors need to address the following concerns:

Major concerns:

1) The authors identified 91 proteins that interact with LINC00493 (Figure 3) and decided to focus on PABPC4. Why? Was a potential impact of NCL on SMIM26 analysed? What about the most strongly enriched candidate? What is the name of this protein and was it functionally analysed?

2) The authors argue that PABPC4, via binding to LINC00493, regulates translation of SMIM26. Indeed, knockdown of PABPC4 decreases SMIM26 expression in two cell lines (Fig. 3C), seemingly without affecting RNA expression of LINC00493/SMIM26 (Fig. 3D). While this suggests a disturbed translation of SMIM26 upon PABP4 depletion, it is also possible that SMIM26 protein turnover is altered. Thus, the authors should analyze microprotein stability (CHX treatments) in control and PABPC4-depleted cells.

3) The authors claim that the interaction between AGK and SMIM26 increases the amount of AGK in the mitochondria by preventing its transport to the cytosol. The data shown in Fig.6J are not really convincing nor consistent between both cell lines and lack the reverse (loss-of-function) experiment. Therefore, please provide a quantification of AGK protein levels in mitochondria and the cytosol in control and SMIM26 overexpressing cells normalized to COXIV and tubulin levels, respectively. Also, the authors should investigate AGK localization in LINC00493/SMIM26-depleted cells.

4) In Figures 6L, M, O the authors show that AGK overexpression can revert SMIM26 effects on AKT phosphorylation as well as cell migration, invasion, and OCR rates. According to their model (Fig.7), AKT is the key downstream factor in this pathway. Please show that the AGK-mediated reversion depends on AKT. For example, the authors could knockdown AKT or use an AKT inhibitor.

Minor concerns:

5) Overall survival analysis is presented in Figure 2C using a 30:70 sample split. Please repeat analysis using 50:50 (median split) and / or upper 25% vs. lower 25%.

6) The authors state that "We next performed RNA pulldown coupled with stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometric analysis (RNA pulldown-SILAC-MS) to identify the post-transcriptional regulators of SMIM26". The authors still need to explain the rational of the SILAC approach and the selection of the two cell lines in more detail. Why was a SILAC method chosen?

7) The authors developed an antibody against SMIM26 and performed IHC analyses in a large panel of tumor (T) and normal (N) tissues (see Fig.2B). Yet, in Figure 3G they limit their PABPC4-SMIM26 co-expression analysis to four T/N pairs only. The

authors should perform IHC analyses in order to detect PABPC4 in their TMA and perform correlation analyses. Alternatively, the authors should leverage the CPTAC data and perform the respective correlation analysis.

8) The authors suggest that SMIM26 overexpression prevents EMT given its impact on Vimentin (see Fig.4F). Is E-cadherin expression altered as well?

9) What is depicted in Fig.S7B? The figure is not intuitive and does not allow one to draw conclusions about pathway enrichment/depletion (statistics?). What are the individual lines/columns? Please provide a more convincing presentation of this KEGG pathway analysis.

10) Please provide patient sample information and ethical statement for analyses shown in Fig.2.

Further suggestions:

11) Please carefully check the manuscript and perform language editing, if needed.

12) In the discussion the authors mention that SMIM26 could be a target to treat cancers. However, SMIM26 is downregulated in cancers (see Figure 2). Thus, "target" might be misleading here. However, restoration of SMIM26 expression would be an option. Moreover, the authors might speculate about AGK inhibitors (alone or in combination with AKT inhibitors).

Referee #3:

In the paper entitled "Mitochondrial Microprotein SMIM26 Encoded by LINC00493 Exerts Antimetastatic Activity in Clear Cell Renal Cell Carcinoma" Meng and colleagues characterize a previously identified micropeptide derived from LINC00493 in ccRCC and show its localization at mitochondria. The paper is timely and research well-conceived, however I still have a few comments.

Major concerns:
-The effect on vimentin in 769-P is not convincing, thus rising the possibility of a cell line specific effect.
-Mouse experiment in Fig.4G is missing stats and "The Key control" (mutant control not producing SMIM26) to be able to claim that "SMIM26 and not LINC00493 suppresses ccRCC growth and metastasis".
-When testing different mutants of SMIM26 I am a bit surprised that the author missed again a key negative control: the protein missing the N-term that they claim is essential for binding to SLC25A11 and/or a fusion protein with a tag at the N-term, as such the protein should not be able to localize to the mitochondria anymore. Regarding this, has SMIM26 a mitochondrial localization signal?
-One major concern is that many experiments have been run on overexpression conditions. For instance, the complex between AGK and SCL25A11 should be probed in KD settings or in cells not expressing SMIM26, to be able to claim that SMIM is bridging these activities.
-There is no link between the in vivo phenotype and the identified protein partners of SMIM26. The co-IP could have been done on xenograft samples and/or immunohistochemistry for phosphor-AKT performed on mouse samples.

Minor:
-In the abstract the authors say: "Especially, the lncRNA-derived mitochondrial microproteins are rarely investigated." This is inaccurate. Actually, localization of many of these micropeptides at mitochondria was one of the first observations following their discovery and this even raised the concern that mito localization could be an artifact of their overexpression and/or of their tagging.
-Title of the first paragraph in the results section: "SMIM26 is a microprotein endogenously encoded by LINC00493 and localizes in mitochondria". It is not clear to me what endogenously means here.
-Last paragraph before discussion: "We then performed RNA-seq in ACHN cells to disclose the downstream pathway through which SMIM26 inhibits cell migration." Here it should be indicated that the RNAseq was run the overexpressing cells, as compared to the knock down mentioned afterwards, to avoid confusion.

Responses to the reviewers' comments

Referee #1:

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Response: Thank you for your constructive comments. According to the suggestions, we firstly examined whether SMIM26 formed dimer. The results of Native-PAGE using purified SMIM26-His recombinant protein did not present SMIM26 dimer (Fig. A-B below).

Next, we detected the minimal SMIM26 motifs required for the binding of AGK and SLC25A11. Plasmids encoding 1-13 aa (N1) or 14-35 aa (N2) of N-ter of SMIM26 protein were constructed (Fig. C-D below). The results of Co-IP indicated that SMIM26 interacted with AGK and SLC25A11 through N2 and N1, respectively (Fig. E below) [Figures for referees not shown.]. Our results suggest that SMIM26 was capable of enhancing the interaction of AGK and SLC25A11.

2. The authors suggest that RNA binding protein PABPC4 regulates SMIM26 expression. Although the levels of PABPC4 in ccRCC are lower than the adjacent normal tissues, however, the authors did not show that PABPC4 expression is lower in ACSN than that in HEK293 cells. In Fig.3C, the authors have examined the levels of PABPC4 in these two cell lines individually, they need run the samples in the same gel to compare the levels of PABPC4 in these cell lines directly.

Response: Thank you. The result of western blotting showed that the expressions of SMIM26 and PABPC4 in ACHN were lower than those in HEK293T. The results are shown in the figure below[Figures for referees not shown.], and please refer to Fig.3E of our revised manuscript.

3. The authors claimed that "Blockage on the interaction of SMIM26-SCL25A11 by siRNAs dramatically abrogated the SMIM26-mediated suppression of cell migration and invasion (Fig. 5P and Fig. S6G)". Based on the method they used (siRNA to knockdown SLC25A11), the authors did not provide direct evidence to support that SMIM26 plays a regulative role in mitochondrial function and cell migration by binding to SLC25A11. Rescue experiment with full length and mutant SCL25A11 not binding with SMIM26 might be helpful.

Response: Thank you for your constructive suggestions. According to the structural

annotation of SLC25A11 in UniPort (https://www.uniprot.org/), three plasmids encoding truncated SLC25A11 protein lacking R1, R2 or R3 were constructed (Fig. A below). Co-IP experiments, using specific antibodies of flag and Myc, indicated that SLC25A11 interacted with SMIM26 through R1 domain (Fig. B below).

Next, rescue experiments with full length and ΔR1 SCL25A11 were performed in SMIM26-overexpressing ccRCC cells. Re-expression of full length SCL25A11, but not ΔR1 SLC25A11, markedly restored SLC25A11 siRNA-mediated rescue of cell migration and invasion in ACHN and 769-P cells with ectopic SMIM26 (Fig. C-D below)[Figures for referees not shown.] suggest that SMIM26 exerts anti-metastatic activity via interaction with SLC25A11.

4. Based on the proposed model that SMIM26 retains AGK in mitochondria to inhibit its capability of catalyzing AKT phosphorylation, while overexpression of AGK in SMIM26-expression cells rescued the AKT phosphorylation, the authors are recommended to examine whether the levels of AGK in cytoplasm (outside of mitochondria) and PTEN phosphorylation are increased when AGK is overexpressed in SMIM26-expression ccRCC cells.

Response: Thank you. AGK has been implicated in cancer by phosphorylating PTEN at Thr382/383 to block its phosphatase activity [1,2]. Our supplementary data showed that phosphorylation of AKT and PTEN was decreased by SMIM26 overexpression, but was increased by AGK overexpression. Ectopic expression of AGK resulted in restoration of SMIM26-reduced PTEN phosphorylation at Thr382/383 (Fig. A below). On the other hand, the expression of cytosolic AGK was markedly increased when AGK was overexpressed in SMIM26-expressing ccRCC cells (Fig. B below)[Figures for referees not shown.].

[1] Hu Z, Qu G, Yu X, Jiang H, Teng XL, Ding L, Hu Q, Guo X, Zhou Y, Wang F, Li HB, Chen L, Jiang J, Su B, Liu J, Zou Q. Acylglycerol Kinase Maintains Metabolic State and Immune Responses of CD8+ T Cells. Cell Metabolism. 2019 Aug 6;30(2):290-302.e5. doi: 10.1016/j.cmet.2019.05.016.

[2] Ning N, Zhang S, Wu Q, Li X, Kuang D, Duan Y, Xia M, Liu H, Weng J, Ba H, Tang Z, Cheng X, Mei H, Huang L, Ao Q, Wang G, Hu Y, Laurence A, Wang J, Wang G, Yang XP. Inhibition of acylglycerol kinase sensitizes DLBCL to venetoclax via upregulation of FOXO1-mediated BCL-2 expression. Theranostics. 2022 Jul 18;12(12):5537-5550. doi: 10.7150/thno.72786.

Minor points:

1, In Fig.1F, The quantification result was not provided.

Response: Thank you for your comments. The quantification result has been provided in revised Fig. 1F.

2, In Fig. 1H & 1J, Fig. 3C & 3E & 3G, etc, The western blots lack molecular weight labels.
Response: Thank you for pointing out our omissions. The molecular weight labels have been provided in the related figures of our revised manuscript.

3, SMIM26-mut-flag (start codon ATG was mutated to GGA and stop codon TAA was mutated to GCT) plasmids. Why the stop codon in the SMIM26-mut-Flag is also mutated? Is it necessary?
Response: Thank you for your comments. In our study, we mutated the stop codon in the SMIM26-mut-Flag to prevent the translation of SMIM26 short abnormal variants, thus ensuring that the overexpressed RNA would not be translated into proteins.

4, In Fig. 4E, how is the intensity of F-actin fluorescence quantified? The method part did not give a detailed description.
Response: Thank you. The description of F-actin staining and quantification has been supplemented in the Materials and Methods section of our revised manuscript.

5, In Fig. 5I, The signals of immunoprecipitated Mut1 & Mut2 are much lower than the Full-length SMIM26. A better representative image is required.
Response: Thank you. We have redone the detection and provided better images in revised Figure 5I.

Referee #2:

Declaration: I was involved in a previous evaluation process of this manuscript for another journal and provide my updated suggestions herein.

In the current manuscript Kun Meng and colleagues describe the expression and function of a small microprotein called SMIM26, which is encoded by the putative non-coding RNA LINC00493. The authors convincingly show that SMIM26 is expressed in a panel of human (cancer) cell lines. SMIM26 localizes to mitochondria and probably is inserted in the mitochondrial membrane. Using a custom-made anti-SMIM26 antibody that authors investigated the expression of this microprotein in a panel of normal and cancerous human tissues. They could detect a significant downregulation of SMIM26 protein, but not LINC00493 expression in kidney cancer (ccRCC). Since the expression of the SMIM26 microprotein and its host RNA showed a poor correlation in cells and tissues, the authors tried to identify post-transcriptional regulators of SMIM26. RNA pulldown and RIP assays revealed an interaction between LINC00493 and PABPC4 and knockdown of the latter reduced SMIM26 protein levels. Further cellular and molecular assays establish SMIM26 as an inhibitor of kidney cancer cell migration, invasion and metastasis potentially via its interaction with SLC2511 and AGK. These interactions enhance GSH import into mitochondria, decrease the fraction of AGK in the cytosol and thereby reduce AKT phosphorylation.

In summary, the authors performed a comprehensive panel of in vitro and in vivo experiments and could establish a critical function for a novel microprotein regulating cancer cell motility in a human cancer entity. Therefore, this study is relevant and important as it sheds light into a new and understudied fraction of the human proteome encoded by recently suggested non-coding transcripts.
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Response: Thank you for your comments. We performed RNA pulldown-SILAC-MS in HEK293T and ACHN cells to identify the potential post-transcriptional regulators of SMIM26. A total of 91 proteins bound to LINC00493 were identified by three biological replicates. Compared with ACHN cells, the binding capabilities of 25 RNA-binding proteins (RBPs) were enhanced in HEK293T cells. When these 25 RBPs were subjected to the protein-RNA interactions software catRAPID, two candidates (PABPC4 and Nucleolin (NCL)) were predicted as LINC00493 regulators.

Nucleolin is an evolutionarily conserved RBP that plays crucial roles in multiple

molecular processes. It is essential for ribosomal biogenesis, ribosomal RNA (rRNA) transcription and processing, and assembly of ribosomes. Nucleolin also regulates multiple steps in messenger RNA (mRNA) processing, including splicing, stabilization, nucleus-cytoplasmic transport, and translation. Nucleolin is also implicated in the synthesis of microRNAs, the proliferation and survival of mammalian cells and self-renewal of embryonic stem cells. (Please refer to reference [1-3])

We next determined the effect of PABPC4 or NCL on the translation of LINC00493. Knockdown of PABPC4, but not NCL, significantly suppressed SMIM26 protein expression (Fig. A below). Therefore, PABPC4 was chosen for further investigations. Our data showed that PABPC4 was able to bind to LINC00493, but did not affect its mRNA expression (Fig. B-C below). The binding of PABPC4 and LINC00493 resulted in the increase of translation of LINC00493 into SMIM26 protein (Fig. D below)[Figures for referees not shown.] .

[1] Liu X, Mei W, Padmanaban V, Alwaseem H, Molina H, Passarelli MC, Tavora B, Tavazoie SF. A pro-metastatic tRNA fragment drives Nucleolin oligomerization and stabilization of its bound metabolic mRNAs. Mol Cell. 2022 Jul 21;82(14):2604-2617.e8. doi: 10.1016/j.molcel.2022.05.008.
[2] Santos T, Salgado GF, Cabrita EJ, Cruz C. Nucleolin: a binding partner of G-quadruplex structures. Trends Cell Biol. 2022 Jul;32(7):561-564. doi: 10.1016/j.tcb.2022.03.003.
[3] Abdelmohsen K, Gorospe M. RNA-binding protein nucleolin in disease. RNA Biol. 2012 Jun;9(6):799-808. doi: 10.4161/rna.19718.

2) The authors argue that PABPC4, via binding to LINC00493, regulates translation of SMIM26. Indeed, knockdown of PABPC4 decreases SMIM26 expression in two cell lines (Fig. 3C), seemingly without affecting RNA expression of LINC00493/SMIM26 (Fig. 3D). While this suggests a disturbed translation of SMIM26 upon PABP4 depletion, it is also possible that SMIM26 protein turnover is altered. Thus, the authors should analyze

microprotein stability (CHX treatments) in control and PABPC4-depleted cells.

Response: Thank you for your constructive suggestions. According to the suggestion, CHX treatment was applied to determine the stability of SMIM26 upon PABPC4 silencing. As shown by the Figure below[Figures for referees not shown.], the depletion of PABPC4 by siRNA markedly expression of SMIM26, but was not able to modulate the protein stability of SMIM26. The half-life of SMIM26 protein was not affected by PABPC4 knockdown in HEK293T cells.

3) The authors claim that the interaction between AGK and SMIM26 increases the amount of AGK in the mitochondria by preventing its transport to the cytosol. The data shown in Fig.6J are not really convincing nor consistent between both cell lines and lack the reverse (loss-of-function) experiment. Therefore, please provide a quantification of AGK protein levels in mitochondria and the cytosol in control and SMIM26 overexpressing cells normalized to COXIV and tubulin levels, respectively. Also, the authors should investigate AGK localization in LINC00493/SMIM26-depleted cells.

Response: Thank you for your constructive comments. We have reperformed the related experiments and replaced Fig. 6J with updated results. As shown in Fig. A below, statistical analyses indicated that SMIM26 overexpression significantly increased mitochondrial AGK, but decreased cytosolic AGK.

As suggested, AGK localization was further examined in SMIM26-depleted cells. We found that deletion of SMIM26 inhibited AGK accumulation in mitochondria (Fig. B below)[Figures for referees not shown.]. These data suggest that SMIM26 modulates the cellular localization of AGK.

4) In Figures 6L, M, O the authors show that AGK overexpression can revert SMIM26 effects on AKT phosphorylation as well as cell migration, invasion, and OCR rates. According to their model (Fig.7), AKT is the key downstream factor in this pathway. Please show that the AGK-mediated reversion depends on AKT. For example, the authors could knockdown AKT or use an AKT inhibitor.

Response: Thank you for your constructive comments. AKT inhibitor MK-2206 was used to determine the role of AKT activation in SMIM26-mediated phenotypes. The results of western blotting and transwell assays demonstrated that the reverse effect of AGK overexpression on SMIM26 can be inhibited by AKT inhibitor. The treatment of MK-2206 markedly inhibited the phosphorylation of AKT in ccRCC cells with SMIM26 and AGK overexpressions, and suppressed the AGK-mediated reversion of cell migration and invasion (Fig. A-B below)[Figures for referees not shown.].

Minor concerns:

5) Overall survival analysis is presented in Figure 2C using a 30:70 sample split. Please repeat analysis using 50:50 (median split) and / or upper 25% vs. lower 25%.

Response: Thank you for your constructive comments. Accordingly, we have redone the Kaplan-Meier analyses, using upper 25% vs. lower 25%. Patients with low SMIM26 expression were accompanied with worse prognosis, compared with those with high SMIM26 expression. Please refer to the figure below [Figures for referees not shown.] or our revised Figure 2C.

6) The authors state that "We next performed RNA pulldown coupled with stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometric analysis (RNA pulldown-SILAC-MS) to identify the post-transcriptional regulators of SMIM26". The authors still need to explain the rational of the SILAC approach and the selection of the two cell lines in more detail. Why was a SILAC method chosen?

Response: Thank you. Stable isotope labeling by amino acids in cell culture (SILAC) is a quantitative proteomic technique based on the incorporation of normal essential amino acids (light label) and isotopic modified amino acids (heavy label) into cell culture, leading to light- and heavy-marked protein synthesis. SILAC has been widely used in the identification of RNA binding proteins (RBPs) that is involved in protein translation [1-6]. Here we applied SILAC to screen for potential regulators of SMIM26 translation by quantitatively comparing the binding capacity of LINC00493 and RBPs in HEK293T and ACHN cells. We have supplemented this information in our revised manuscript.

[1] Nuria CV, Sergi S, Lara PM, Marion S, Falk B. The RNA fold interactome of evolutionary conserved RNA structures in S. cerevisiae. Nature Communications. 2020 Jun;11(1):2789. doi: 10.1038/s41467-020-16555-4.

[2] Séverine B, Yasmeen A, Laura TM, Céline V, Andy C, Peter G, Edouard B, Mark W, Angus I L. Establishment of a protein frequency library and its application in the reliable identification of specific protein interaction partners. Mol Cell Proteomics. 2010 May;9(5):861-879. doi: 10.1074/mcp.M900517-MCP200.

[3] Kugeratski FG, Hodge K, Lilla S, McAndrews KM, Zhou X, Hwang RF, Zanivan S, Kalluri R. Quantitative proteomics identifies the core proteome of exosomes with syntenin-1 as the highest abundant protein and a putative universal biomarker. Nat Cell Biol. 2021 Jun;23(6):631-641. doi: 10.1038/s41556-021-00693-y.

[4] Schäfer JA, Bozkurt S, Michaelis JB, Klann K, Münch C. Global mitochondrial protein import proteomics reveal distinct regulation by translation and translocation machinery. Mol Cell. 2022 Jan 20;82(2):435-446.e7. doi: 10.1016/j.molcel.2021.11.004.

[5] Kurosaki T, Mitsutomi S, Hewko A, Akimitsu N, Maquat LE. Integrative omics indicate FMRP sequesters mRNA from translation and deadenylation in human neuronal cells. Mol Cell. 2022 Dec 1;82(23):4564-4581.e11. doi: 10.1016/j.molcel.2022.10.018.

[6] Cox J, Matic I, Hilger M, Nagaraj N, Selbach M, Olsen JV, Mann M. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. Nat Protoc. 2009;4(5):698-705. doi: 10.1038/nprot.2009.36.

(7) The authors developed an antibody against SMIM26 and performed IHC analyses in a large panel of tumor (T) and normal (N) tissues (see Fig.2B). Yet, in Figure 3G they limit their PABPC4-SMIM26 co-expression analysis to four T/N pairs only. The authors should perform IHC analyses in order to detect PABPC4 in their TMA and perform correlation analyses. Alternatively, the authors should leverage the CPTAC data and perform the respective correlation analysis.

Response: Thank you for your suggestion. As suggested, the correlation between SMIM26 and PABPC4 has been evaluated in both CPTAC and SYSUCC cohorts. Positively correlations have been found and indicated in our revised manuscript (Fig. A-B below) [Figures for referees not shown.].

8) The authors suggest that SMIM26 overexpression prevents EMT given its impact on Vimentin (see Fig.4F). Is E-cadherin expression altered as well?

Response: Thank you. The expressions of EMT markers including Fibronectin, E-cadherin and Vimentin have been detected in ccRCC cells with SMIM26 overexpression or depletion (Fig. A-B below)[Figures for referees not shown.] .

(9) What is depicted in Fig.S7B? The figure is not intuitive and does not allow one to draw conclusions about pathway enrichment/depletion (statistics?). What are the individual lines/columns? Please provide a more convincing presentation of this KEGG pathway analysis.

Response: As suggested, graph images of KEGG pathway analysis have been replaced. Please refer to the following figure [Figures for referees not shown.] or our revised Appendix Fig. S8B.

10) Please provide patient sample information and ethical statement for analyses shown in Fig.2.

Response: Thank you. The information of patients enrolled in our study has been provided in our revised Appendix Table S1. Ethical statement was supplemented in the Materials and Methods section of our revised manuscript.

Further suggestions:

11) Please carefully check the manuscript and perform language editing, if needed.

Response: Thank you. We have carefully gone through our manuscript. Some grammar or type errors have been corrected.

12) In the discussion the authors mention that SMIM26 could be a target to treat cancers. However, SMIM26 is downregulated in cancers (see Figure 2). Thus, "target" might be misleading here. However, restoration of SMIM26 expression would be an option. Moreover, the authors might speculate about AGK inhibitors (alone or in combination with AKT inhibitors).

Response: Thank you. We have corrected the related errors in the discussion of the revised manuscript.

Referee #3:

In the paper entitled "Mitochondrial Microprotein SMIM26 Encoded by LINC00493 Exerts Antimetastatic Activity in Clear Cell Renal Cell Carcinoma" Meng and colleagues characterize a previously identified micropeptide derived from LINC00493 in ccRCC and show its localization at mitochondria. The paper is timely and research well-conceived, however I still have a few comments.

Major concerns:
-The effect on vimentin in 769-P is not convincing, thus rising the possibility of a cell line specific effect.
Response: Thank you for your constructive comments. To determine whether EMT was involved in SMIM26-mediated suppression of cell migration, we performed western blotting to examine the expressions of EMT markers including Fibronectin, E-cadherin and Vimentin. As shown in Fig. A-B below[Figures for referees not shown.], the expressions of Vimentin and Fibronectin were significantly downregulated by SMIM26 overexpression, and increased in cells with SMIM26 depletion. E-cadherin was increased in SMIM26-overexpressing cells. These data suggest that EMT was inhibited by the ectopic expression of SMIM26.

-Mouse experiment in Fig.4G is missing stats and "The Key control" (mutant control not producing SMIM26) to be able to claim that "SMIM26 and not LINC00493 suppresses ccRCC growth and metastasis".
Response: Thank you. According to the suggestion, we have reperformed the *in vivo* experiments. As shown in Fig. A-F [Figures for referees not shown.], SMIM26, but not blank control nor mutant control, markedly inhibited the tumor growth and metastasis in ccRCC.

-When testing different mutants of SMIM26 I am a bit surprised that the author missed again a key negative control: the protein missing the N-term that they claim is essential for binding to SLC25A11 and/or a fusion protein with a tag at the N-term, as such the protein should not be able to localize to the mitochondria anymore. Regarding this, has SMIM26 a mitochondrial localization signal?

Response: Thank you. As suggested, we have used a canonical signal peptide prediction software of SignalP to find that no mitochondrial localization signal was available in SMIM26. For the reviewer's concern, we actually constructed plasmids encoding flag-fused SMIM26 protein lacking N-terminal (Δ1-35aa, Mut2) (Fig. A-B below). However, Mut2 protein was not detectable, using antibodies of flag (Fig. C below). Instead, we detected the minimal SMIM26 motifs required for the binding of AGK and SLC25A11. Plasmids encoding 1-13 aa (N1) and 14-35 aa (N2) of N-terminal of SMIM26 protein were constructed (Fig. D below). Cellular fractionation experiments demonstrated that N2, but not N1, localized in mitochondria (Fig. E below)[Figures for referees not shown.]. The results of Co-IP indicated that SMIM26 interacted with AGK and SLC25A11 through N2 and N1, respectively (Fig. F below). These data have been supplemented in our revised manuscript.

-One major concern is that many experiments have been run on overexpression conditions. For instance, the complex between AGK and SCL25A11 should be probed in KD settings or in cells not expressing SMIM26, to be able to claim that SMIM26 is bridging these activities.

Response: Thank you for your constructive comments. According to the reviewer's suggestion, we have examined the binding ability of AGK with SLC25A11 in ccRCC cells with SMIM26 knockdown. The depletion of SMIM26 noticeably reduced the binding of AGK and SLC25A11 (Fig. below [Figures for referees not shown.] or Fig. 6L in the revised manuscript).

-There is no link between the in vivo phenotype and the identified protein partners of SMIM26. The co-IP could have been done on xenograft samples and/or immunohistochemistry for phosphor-AKT performed on mouse samples.

Response: Thank you. The phosphorylation of AKT at Ser473 was examined in xenograft tumor samples (Fig. A below), showing that AKT phosphorylation was decreased in tumors with SMIM26 overexpression, as compared with NC and Mut groups. Moreover, we also performed Co-IP assay, using proteins extracted from xenograft tumor tissues. The results indicated that SMIM26 formed protein complex with AGK and SLC25A11 (Fig. B below) [Figures for referees not shown.] .

Minor:

-In the abstract the authors say: "Especially, the lncRNA-derived mitochondrial microproteins are rarely investigated." This is inaccurate. Actually, localization of many of these micropeptides at mitochondria was one of the first observations following their discovery and this even raised the concern that mito localization could be an artifact of their overexpression and/or of their tagging.

Response: We thank the reviewer's concern. We have deleted the related sentence in our revised abstract.

-Title of the first paragraph in the results section: "SMIM26 is a microprotein endogenously encoded by LINC00493 and localizes in mitochondria". It is not clear to me what endogenously means here.

Response: Thank you. We have deleted "endogenously" in the related sentence.

-Last paragraph before discussion: "We then performed RNA-seq in ACHN cells to disclose the downstream pathway through which SMIM26 inhibits cell migration." Here it should be indicated that the RNA-seq was run the overexpressing cells, as compared to the knock down mentioned afterwards, to avoid confusion.

Response: Thank you. We have corrected the related sentence to "We then performed RNA-seq in SMIM26-overexpressing ACHN cells to disclose the downstream pathway through which SMIM26 inhibited cell migration.".

Dear Dr. Zhang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from two of the three referees that I have asked to re-evaluate your study, you will find below. Referee #3 declined to look into the revision. However, going through your point-by-point response, I consider his/her points as adequately addressed. As you will see, the remaining two referees now fully support the publication of your study in EMBO reports.

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The manuscript is suitable for publication in EMBO reports without further revision.

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The authors fully addressed my concerns. I do not have any further questions.

The authors have addressed all minor editorial requests.

Chris Zhiyi Zhang
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	