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PTENε suppresses tumor metastasis through regulation of filopodia formation

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1st Editorial Decision 15th Jul 2020

Dear Dr Yin,

Thank you for your interest and for the submission of your manuscript (EMBOJ-2020-105806) to The EMBO Journal, as well as for your patience with our response, which got delayed due to protracted referee inoput as well as detailed discussions in the team. Your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

The referees acknowledge the potential interest of your results, although they also express major concerns. In particular, referee #1 raises substantial issues regarding the claims made on unique dependence of PTEN-epsilon on eIF2a (Ref#1, pts. 4,5). Referee #2 is concerned that the endogeneous relevance of plasma membrane localized PTEN-epsilon is only prematurely supported and requires additional experimentation (ref#2, pts. 3,5,7; see also ref#3, pt.6). In addition, this referee points to the need to consolidate epsilon-specific roles in VASP-mediated filopodia formation (ref#2, pts.6,7). Further, the referees raise a number of issues related to missing controls, methods annotation, statistics and data illustration, discussion of results and literature as well as wording matters that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I need to stress though that we do need strong support from the referees on a revised version of the study in order to move on to publication of the work and as to the open outcome of the revisional work suggest to keep EMBO Reports in mind for this work as an alternative venue.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

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.

I this context I also want to point to our adjusted GTA 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 us at any time to discuss an adapted revision plan for your manuscript should you need additional time, and also if you see a paper with related content published elsewhere.

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/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 & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). 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])

Our journal also 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 https://www.embopress.org/page/journal/14602075/authorguide#referencesformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 13th Oct 2020.

Link Not Available

Referee #1:

In this manuscript, Zhang et al. identifies a new isoform of PTEN, PTEN-epsilon, which possesses a unique function in suppressing cell migration and invasion. In addition, they show that PTEN-epsilon is translated from an upstream CUG codon within PTEN 5'UTR. The authors provide convincing evidence for the presence of PTEN-epsilon and its function in tumor suppression. This is an interesting manuscript that highlights the importance of translation control and alternative starting codons in regulating the protein levels of key tumor suppressors. Overall, this manuscript is suitable for publication in EMBO Journal, however there are several major points below that need to be addressed in order to strengthen the significance of the findings supporting the authors' claims.

1. In Figures 1 and 2D-F, what are the bands slightly below PTEN-beta and above PTEN-epsilon? They seem to disappear upon UCU783 mutation. Are they other unidentified isoforms of PTEN-long, or are they the modified/cleaved version of PTEN-alpha and beta?

2. In the text for Figure 2D, "UCG783" should be "UCU783". Also, please make sure all the numbers of the starting codons are listed correctly.

3. The author states, "to determine the effect of eIFs on CUG initiated PTENε, we first examined the expression of eIF5 and eIF2A in a panel of cancer cell lines as listed in Fig. 1C". "Fig. 1C" should be changed to "Fig. 1B".

4. In Figure 4B, there are reductions in all the isoforms (alpha, beta and epsilon) of PTEN when eIF2A is knocked down. Similarly, eIF2A overexpression also renders an increase in all isoforms. This is confusing due to the fact that if eIF2A is responsible for the CUG-mediated translation initiation, why is the upstream beta isoform (initiated by the AUU) also affected?

Can the authors provide a qPCR data for the PTEN mRNA expression upon eIF2A overexpression/knockdown?

Also, alterations in the upstream starting codon may affect the efficiency of the downstream mRNA isoforms. Therefore, when eIF2A is knocked down, do the authors observe an increase in the canonical PTEN? Similarly, when eIF2A is overexpressed, is there a reduction in the canonical PTEN? It would be great if the authors could provide lower exposures of the western blot analysis and quantification to address these questions.

5. In relation to the point above, it is very intriguing that eIF2A can affect the translation efficiency of specific isoforms, however the authors should at least strengthen this point by using reporter assays. For example, the authors should perform a reporter assay employing the 5'UTR of PTEN, where all the initiation codons of PTEN isoforms are in frame with the firefly coding frame mRNA, and test how overexpressing or knocking down eIF2A can affect the translation of distinct isoforms initiated by specific upstream codons.

6. The authors need to clarify how the putative hairpin depicted in Figure 4D was generated. If it is only a prediction, this needs to be clearly stated. If it is, indeed, a predicted structure, it might be misleading to add it in the primary figures and should be placed in the SOM.

7. In Figure 5A, the authors aim to specifically overexpress the PTEN-alpha isoform. However, as this mRNA also contains the starting codon for beta and epsilon, the question is whether they are overexpressing all the isoforms at the same time. Did the authors mutate the starting codon for beta and epsilon in order to specifically overexpress alpha? The same controls should be taken into consideration for PTEN-beta overexpression.

8. The authors present an N-terminus folding prediction for PTEN-epsilon. Do the PTEN-alpha and beta isoforms that contain longer N-terminals fold differently?

9. It is interesting that the authors show in Figure 7E-F that deleting only the PTEN-epsilon can increase cell motility. However, to strengthen the claim on the functional activity for this specific PTEN isoform, can the authors also include additional controls, such as overexpression of the other PTEN isoforms?

Referee #2:

In this manuscript, Yin's group identified a novel N-terminal-extended PTEN isoform designated as PTENε, of which the translation is initiated from the CUG816 codon within the 5'UTR region of PTEN mRNA. They also revealed that PTENε mainly localizes in the cell membrane, and dephosphorylates VASP and ACTR2. Overexpression of PTENε reduces filopodia formation and inhibits the metastasis of tumor cells. These original findings are interesting and suitable to be published in EMBO J, but some concerns should be addressed.

1.In figure 3D, they should also blot these precipitates with anti-PTEN antibody which show canonical PTEN besides its long isoforms.
2. They described that the level of eIF2A transcripts and protein positively correlated with PTENε protein in Figure 4A, but they actually only detected transcripts rather than proteins of two kinds of eIFs. I propose that they should also compare with correlation of their proteins.
3.Whether does endogenous PTENε inhibit phosphorylation of VASP and ACTR2? To see the endogenous role of PTENε, they should detect the levels of VASP and ACTR2 phosphorylation in PTENε knockout cells. They should also use phosphatase-deficient mutants as negative controls in Figure 4H/I, and use PTEN and other isoforms as negative controls in Figure 4G. Also, they should pointed out that overexpression or knockdown of eIF2A also significantly regulated other several long isoforms of PTEN such as PTENα/β besides PTENε expression in Figure 4C/D, while disruption of the palindromic sequence leads to a marked reduction of PTENε rather than PTENα/β expression in Figure 4F. Did these results indicate the same mechanisms to regulated expressions of these PTEN variants?
4.Fig. 5A should show western blot to ensure expression of only a single isoform in each instance.
5. It is hard to understand how PTENε showed different cellular localization from PTENα and PTENβ. It also contains nucleus-localized signal recently identified in PTENα/β. Actually, according to Figure 5B-D, PTENε appears be seen in cytoplasm and nucleus besides on the cell membrane. I also like to know whether PTENε is localized into nuclei by fractionation analysis in Figure 5D. By the way, the cervical cancer cell line Hela is used throughout the whole study. However, according to this reviewer's knowledge, Hela cells do not express E-cadherin (PMID: 12751384; PMID: 7636625). Why did authors detect strong E-cadherin signal in their Hela cells in Figure 5D/G.
6. PTENα/β also own the amino acid sequence of PTENε protein. They should also address whether PTENα/β interacted with VASP, FSCN1, CDC42 and ACTR2 to support their conclusion for the specificity of the interaction of PTENε with these membrane proteins.
7. The authors showed that PTENε played a role in filopodia formation, migration and invasion, but they did not test the involvement of plasma membrane localization or VASP and ACTR2 in these processes. Considering the small amount of PTENε even compared to PTENα and PTENβ, the endogenous role of PTENε should be tested in these processes within the same cell line, for example, B16 cells used for the mouse pulmonary metastasis model.
Minor concerns
8. n Figure 1A, they showed four unidentified proteins of molecular weights lower than PTENβ (between 72 and 55kDa) in Hela cells, which are designated as γ, δ, ε, and ζ isoforms of PTEN. I proposed that it is better to label all these isoforms in all 4 panels of Figure 1.
9. There are too many mistakes when describing their results and mis-spelling. For examples, legend descriptions are inconsistent with some Figure, such as Figure 4B, 6D. When describing Figure 2F (page 7), lane 5 but not lane 4 should be compared to other lanes; In page 11, a serious of

should be a series of; On page 8, "as listed in Fig. 1C." should be Fig. 1B.On page 12, subtract should be substrate; in Figure 5D/G, tululin should be tubulin. "we first examined the expression of eIF5 and eIF2A in a panel of cancer cell lines as listed in Fig. 1C.", a panel of cancer cell is listed in Fig. 1B, not Fig. 1C.
10.In the introduction, some novel reports on nuclear PTEN should be mentioned and cited such as Nat Commun.2018;9(1):2392;2020;11(1):1720;

Referee #3:

In this manuscript, authors identified PTENε (or PTEN5), as a novel N-terminal-extended PTEN isoform that suppresses tumor invasion and metastasis. They showed that PTENε mainly localizes in the cell membrane and affects filopodia formation and cell motility potentially through VASP and ACTR2 dephosphorylation. They found that its overexpression reduces filopodia formation and inhibits the metastasis capacity of tumor cells.
The concepts raised in the manuscript are interesting and constitute an advance in the understanding of the relevance and diversity of PTEN functions. Authors identified a novel isoform of PTEN with distinct localization and function compared to the known members of the family. Overall, this is a very interesting and timely study for EMBO J. There are general comments and some suggestions that the authors could consider before publication.

General comments:
1. The biochemical evidence of the existence and function of PTENε is convincing. I have no major criticisms with the data presented and the conclusions reached.
2. The regulation of PTENε by eIF2 is intriguing. This protein is profoundly influence by post-translational modifications. It would be interesting to ascertain whether Integrated Stress Responses leading to eIF2A S51 phosphorylation alters the production of this isoform.
3. CRISPR/Cas9 mutants: more information about genotyping approach should be provided, whether the lesion was homozygous/heterozygous, and whether any potential off-targets of the sgRNA were checked.
4. Some results (specifically clarified bellow) and discussion should be revised to avoid overstatements that go far beyond results. For instance, in the discussion authors state: "our data strongly indicate that PTENε exerts this function mainly through regulating the formation of pseudopods". However, results only indicate that filopodia formation is dependent on the phosphatase activity of PTENε (Figure 7a and b).
5. A schematic representation of all the isoforms described would be important (some of the new isoforms are marginally mentioned, but represent an important finding). Also, these new isoforms should be indicated in the relevant panels of figure 1.
6. The mass spectrometry-based determination is interesting. Although challenging, analyzing by mass spectrometry endogenous PTEN immunoprecipitates would be interesting.
7. The filopodia differences in Fig. 7A and B are not obvious. Please, indicate what accounts for filopodia in magnification, and consider including more representative images.
8. In supplementary Fig. 8c, the differences in cell morphology is far from obvious.
Specific comments:
PTEN open-close conformation has also been associated to its activity and localization to membrane. How do the authors reconcile this?
Figure 1a: Authors claimed that they ruled out the possibility that some of these unidentified proteins are cleavage products by performing a PTENα and PTENβ KO. However, at this point of the manuscript, they should mention that it is also possible that those lower MW bands could be modified forms of PTEN (ubiquitinated, phosphorylated...). Also, the authors depict PTENε as a

double-band in the western blot. Can the authors discuss or prove the nature of these bands?
Figure 1b: why are different antibodies used in figure 1a and 1b (polyclonal antibody against full-length PTEN and rabbit monoclonal antibody against the C-terminal region of PTEN, Cell Signaling, 138G6, respectively)?
Figure 1c: Using a PTENα antibody raised in the laboratory, authors identified three of the unidentified proteins with larger molecular weights than PTEN. Any idea about what the forth band on top of PTEN that cannot be identified using this antibody in figure 1c but that is present on figure 1a and b is?
Figure 2a: It would be interesting to see the same western blot probed against PTEN antibody to detect and compare the endogenous levels of PTEN isoforms vs the overexpressed ones.
Figure 3d: Don´t you expect to detect also canonical PTEN in the different tissues? Perhaps the ratio of these forms vs. the canonical PTEN isoform should be presented by blotting with a pan-PTEN antibody.
Figure 4b: here, genetic rescue using reintroduction of FLAG-eIF2A and checking whether you rescue PTEN levels would be useful, although not essential.
Figure 4c: minor spelling correction. Please, correct FALG-eIF5 by FLAG-eIF5 on the first western blot.
Authors concluded from figure 4a-c: "Together, these data indicate that PTENε synthesis is closely regulated by the eIF2A-dependent mechanism." Please, avoid such overstatements that go beyond results since these data only prove that there is a correlation between PTENε and eIF2A levels. More experiments are needed in order to conclude that eIF2A regulates PTENε (i.e. genetic rescue using reintroduction of FLAG-eIF2A).
Figure 6b: Do these proteins (FSCN1, ACTR2, CDC42 and VASP) interact with PTENα and PTENβ? In addition, it would be interesting, although not essential, to perform this IP experiment in PTEN KO HEK 293 cells to discard the fact that the complex that might be formed between the different overexpressed PTEN isoforms and the endogenous ones is responsible for the interaction with FSCN1, ACTR2, CDC42 and VASP.
Figure 6c: this experiment cannot be considered endogenous immunoprecipitation of PTENε since the different PTEN isoforms have been overexpresed. Please, modify this in the text and figure legend. Also, authors concluded from figure 6a-c: "which indicates that these proteins were PTENε targets" Please, avoid such overstatements that go beyond results since these data only prove that FSCN1, ACTR2, CDC42 and VASP interact with PTENε. As a suggestion, the authors could consider performing endogenous IP with anti-PTENα.
Figure 6d: authors claimed "immunofluorescence showed extensive co-localization of PTENε with these interactors in the membrane, whereas PTEN did not (Fig. 6D and Supplementary Fig. 6)". However, authors do not show the immunofluorescence micrographs showing canonical PTEN staining together with the interactors. Furthermore, do the endogenous CDC42, FSCN1, VASP, ACTR2 also colocalize with PTENε? And also, do CDC42, FSCN1, VASP, ACTR2 colocalize with PTENε Y210L?
Figure 6f: Also FSCN1 and CDC42 are reduced. Should be mentioned in the text (end of page 12). Also, the authors ignore these proteins for phosphorylation assays.
Figure 7f: Authors claimed "As shown in Fig. 7F, the number of migrated and invaded cells in Hela PTENε+/- was substantially reduced compared to WT cells". However figure 7f shows that the number of migrated and invaded cells in Hela PTENε+/- was substantially increased compared to WT cells. This should be corrected.
Supplementary Figure 1: It is advised to show the numbers of the nucleotides (position) and not just show the sequence.
Supplementary Figure 4a: Not matched, does have GFP
Supplementary Figure 4a, lower panel in text: Suppl Figure 4b in figure
Supplementary figure 8 a and b: is it consistent that PTENε Y210L is more expressed than PTENε

both in H4 and in Hela cells? Also, in supplementary figure 8b, PTENε G210L should be changed to PTENε Y210L. What does PCDH stand for?
Supplementary figure 8 c and d: it would be interesting to check colony formation in Hela and H4 cells when PTENε Y210L is incorporated. Also, there is a star with uncertain meaning that should be explained.
End of page 12. The authors refer to "substracts": should be substrates

A. Point-by-point responses to the comments by Reviewer #1

In this manuscript, Zhang et al. identifies a new isoform of PTEN, PTEN-epsilon, which possesses a unique function in suppressing cell migration and invasion. In addition, they show that PTEN-epsilon is translated from an upstream CUG codon within PTEN 5'UTR. The authors provide convincing evidence for the presence of PTEN-epsilon and its function in tumor suppression. This is an interesting manuscript that highlights the importance of translation control and alternative starting codons in regulating the protein levels of key tumor suppressors. Overall, this manuscript is suitable for publication in EMBO Journal, however there are several major points below that need to be addressed in order to strengthen the significance of the findings supporting the authors' claims.

We would like to express our sincere thanks to the reviewer for the positive comments on our work. We have addressed each of the comments as follows.

Q1.1. *In Figures 1 and 2D-F, what are the bands slightly below PTEN-beta and above PTEN-epsilon? They seem to disappear upon UCU^{783} mutation. Are they other unidentified isoforms of PTEN-long, or are they the modified/cleaved version of PTEN-alpha and beta?*

R1.1. We thank the reviewer for these constructive comments and we agree that the bands slightly below PTENβ and above PTENε are likely to be other unidentified new isoforms of PTEN.

First, we excluded the possibilities that these bands might be cleaved versions of PTENα and PTENβ. As shown in Fig 1A line 2 versus line 1, the insertion of a TAG stop codon immediately downstream of the PTENβ initiation codon AUU^{594} only led to the disappearance of the slower migrating protein-forms PTENα and PTENβ without any effect on the protein bands slightly below PTENβ and above PTENε, thus ruling out the possibility that some of these unidentified proteins are the cleavage products or modified versions of PTENα and PTENβ.

Besides, a TAG stop codon that terminates translation from upstream initiation sites inserted upstream of the AUG^{1032} start codon of canonical PTEN with CRISPR-Cas9 eliminated these bands slightly below PTENβ and above PTENε without affecting canonical PTEN expression (Fig 1D, lane 2 versus lane 1), excluding the possibility of these unidentified proteins to be the bands of canonical PTEN modifications.

Furthermore, these protein bands slightly below PTENβ and above PTENε can be efficiently recognized by the anti-PTENα antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENα (Fig 1C), indicating that they were more likely to be novel N-terminally extended PTEN variants. However, these new variants of PTEN need further verification through mutational analysis and mass spectrometry, which will be our future direction.

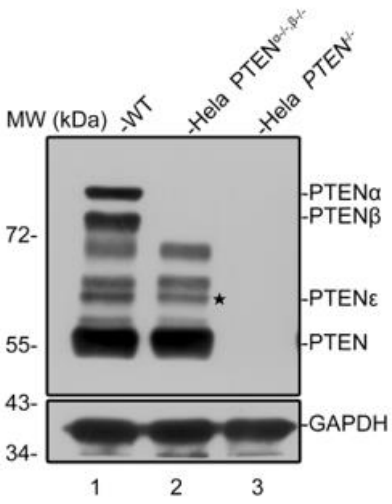


Fig 1A. The discovery of novel PTEN variants in Hela PTEN$^{\alpha-/-, \beta-/-}$ cells. The asterisk shown here and in other immunoblot images throughout this manuscript indicates the corresponding protein band of PTENε.

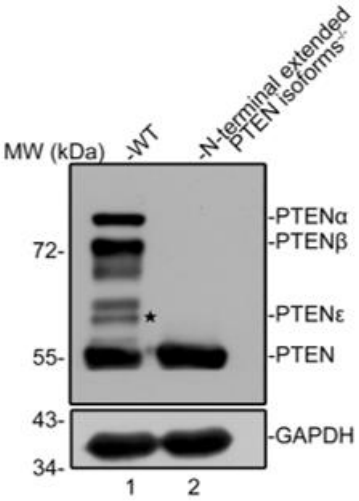


Fig 1D. Disruption of the PTEN mRNA 5'UTR eliminates expression of all PTEN isoforms.

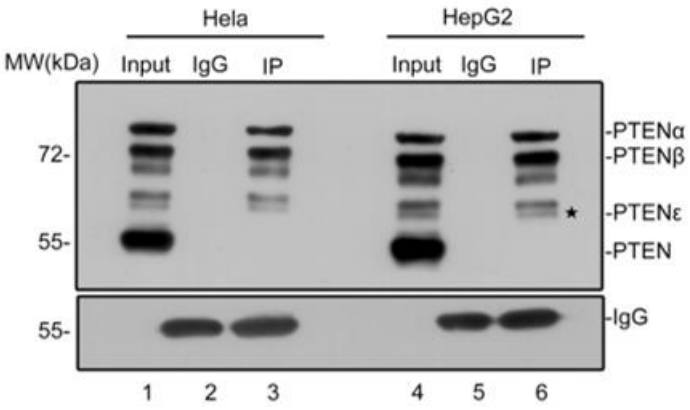


Fig 1C. The endogenous immunoprecipitation in the Hela and HepG2 cancer cell lines by the anti-PTENα antibody.

Q1.2. *In the text for Figure 2D, "UCG^{783}" should be "UCU^{783}". Also, please make sure all the numbers of the starting codons are listed correctly.*

R1.2. We are grateful for the reviewer's careful examination and we apologize for this misspelling. We have prudently checked all these starting codons listed in our manuscript and "UCG^{783}" has been changed to "UCU^{783}" on page 6 in the revised version.

Q1.3. *The author states, "to determine the effect of eIFs on CUG initiated PTENε, we first examined the expression of eIF5 and eIF2A in a panel of cancer cell lines as listed in Fig. 1C". "Fig. 1C" should be changed to "Fig. 1B".*

R.1.3. Our apology for the error. We have carefully looked through all the texts in the manuscript to avoid such mistakes, and "Fig. 1C" has been changed to "Fig 1B" on page 9 in the revised version.

Q1.4. *In Figure 4B, there are reductions in all the isoforms (alpha, beta and epsilon) of PTEN when eIF2A is knocked down. Similarly, eIF2A overexpression also renders an increase in all isoforms.*

a) *This is confusing due to the fact that if eIF2A is responsible for the CUG-mediated translation initiation, why is the upstream beta isoform (initiated by the AUU) also affected?*
b) *Can the authors provide a qPCR data for the PTEN mRNA expression upon eIF2A overexpression/knockdown?*
c) *Also, alterations in the upstream starting codon may affect the efficiency of the downstream mRNA isoforms. Therefore, when eIF2A is knocked down, do the authors observe an increase in the canonical PTEN? Similarly, when eIF2A is overexpressed, is there a reduction in the canonical PTEN? It would be great if the authors could provide lower exposures of the western blot analysis and quantification to address these questions.*

R1.4. We feel very thankful for these constructive comments. According to the suggestions of the reviewer, the following experiments were carried out to address the role of eIF2A in regulation of expression of PTEN variants.

a) We apologize for the reviewer's confusion caused by our inaccurate statement about regulation of eIF2A on the initiation of non-AUG codons. As reported, eIF2A is also responsible for initiation from other non-AUG codons in addition to CUG, such as UUG (Starck *et al*, 2016), which indicates that eIF2A plays an extensive regulatory role in non-AUG initiated translation and thus illustrates the modulation of AUU initiated PTENβ by eIF2A. Accordingly, as shown on page 8, the corresponding statement has been changed to "…Moreover, it is known that eIF2A is crucial for Leu-tRNA initiation at CUG start codons and other non-AUG

codons such as UUG…" in the new revision.

b) As suggested, the relative PTEN mRNA level in eIF2A overexpression or knockdown cells was quantified by real-time fluorescence quantitative PCR. This is shown in Fig R1.1, in which neither knockdown of eIF2A nor overexpression of eIF2A affects relative expression of PTEN mRNA.

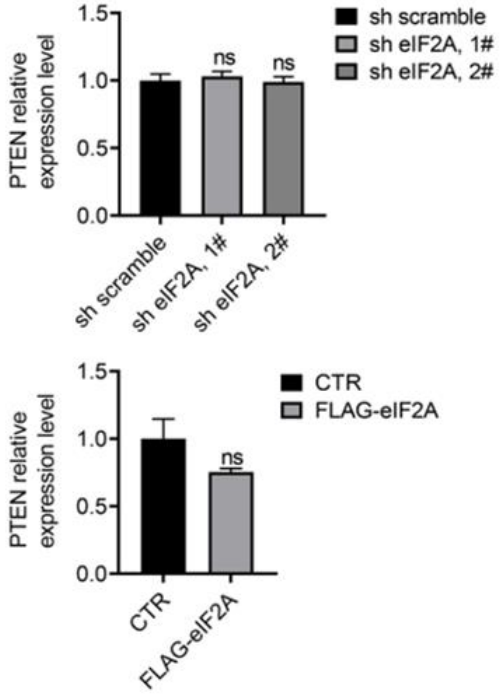


Fig R1.1. Relative expression of PTEN mRNA upon eIF2A overexpression or knockdown.

c) We agree that alterations in the upstream starting codon may affect the initiation efficiency of the downstream mRNA isoforms. To address this concern, we used a PTEN monoclonal antibody (Cell Signaling Technology, 138G6) to detect the protein level of canonical PTEN after eIF2A overexpression or knockdown (Revised Fig EV2C). By analyzing lower exposures of the western blot as suggested, we found that different from the PTEN isoforms initiated from upstream non-AUG codons, expression of endogenous canonical PTEN is negatively related to eIF2A. Overexpression of eIF2A inhibits the translation of canonical PTEN whereas knockdown of eIF2A upregulates expression of canonical PTEN, which is consistent with the previous report by Fuchs' group that the translation landscape shifts from uORFs (upstream open reading frames) within the 5'UTR to ORFs in endogenous eIF2A depleted SCC keratinocytes (Sendoel *et al*, 2017).

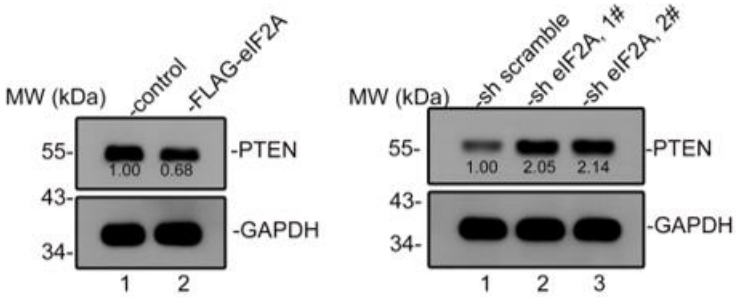


Fig EV2C. Influence of eIF2A alteration on expression of canonical PTEN.

Q1.5. *In relation to the point above, it is very intriguing that eIF2A can affect the translation efficiency of specific isoforms, however the authors should at least strengthen this point by using reporter assays. For example, the authors should perform a reporter assay employing the 5'UTR of PTEN, where all the initiation codons of PTEN isoforms are in frame with the firefly coding frame mRNA, and test how overexpressing or knocking down eIF2A can affect the translation of distinct isoforms initiated by specific upstream codons.*

R1.5. We thank the reviewer for this constructive suggestion. Accordingly, the following experiments were carried out to address the comment.

(a) We linked the 5'UTR of PTEN with luciferase reporter to testify the influence of eIF2A alteration on the translation of distinct isoforms initiated by specific upstream codons. Meanwhile, the non-AUG start codons that initiate other potential PTEN variants within the 5'UTR were mutated to CTC codons to ensure that only one isoform is expressed in each panel. Luciferase activity was measured with the Dual-Luciferase Assay System (Promega) following the manufacturer's protocol. We found that overexpression of eIF2A indeed promotes the translation of PTENα, PTENβ, and PTENε, respectively (Fig EV2D).

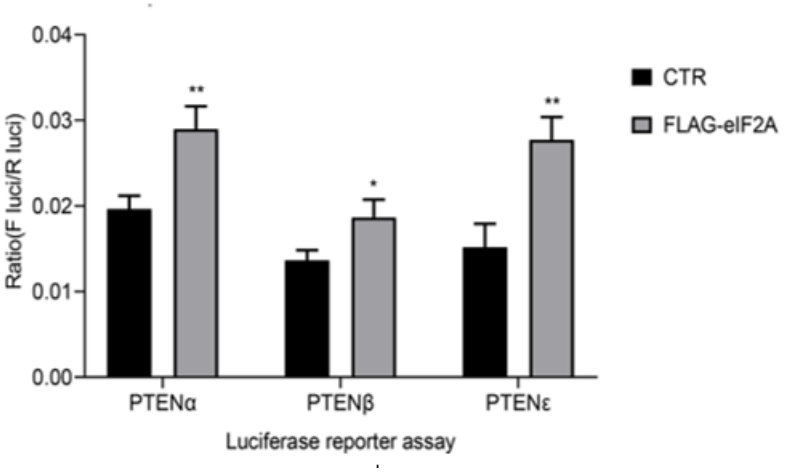


Fig EV2D. Influence of eIF2A alteration on the initiation of individual PTEN isoforms.

(b) As PTENε expression is downregulated markedly in eIF2A knockdown cells, in the revised version, we further measured the level of PTEN isoforms in eIF2A knockdown Hela cells transfected with wildtype FLAG-eIF2A and it turns out that eIF2A overexpression compensates for the downregulation of PTENε and other N-terminal extended PTEN isoforms resulting from knockdown of endogenous eIF2A (Fig EV2B). These results further demonstrate that expression of CUG^{816} initiated PTENε as well as the other two known isoforms PTENα and PTENβ is modulated by eIF2A.

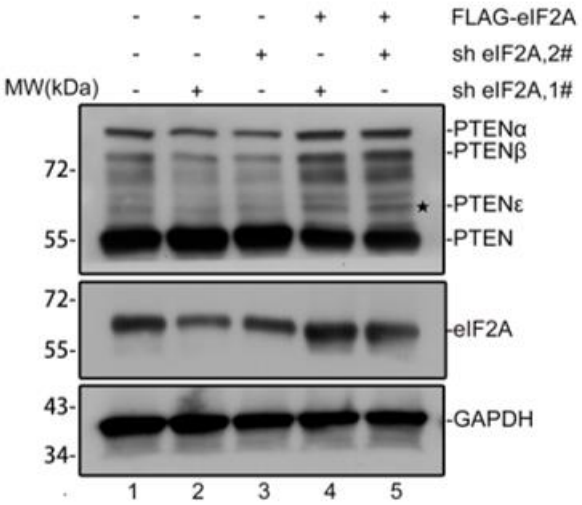


Fig EV2B. The reduced expression of PTEN isoforms in eIF2A knockdown cells was rescued by eIF2A reintroduction.

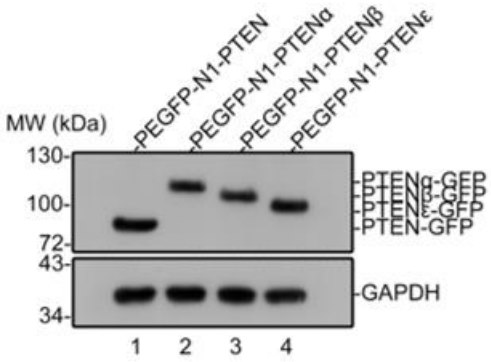
Q1.6. *The authors need to clarify how the putative hairpin depicted in Figure 4D was generated. If it is only a prediction, this needs to be clearly stated. If it is, indeed, a predicted structure, it might be misleading to add it in the primary figures and should be placed in the SOM.*

R1.6. We thank the reviewer for these insightful comments and we apologize for the reviewer's confusion caused by our inadequate statement. The putative hairpin structure shown in Fig 4D and 4E was generated by a web server-Mfold (Zuker, 2003) and this has been clearly depicted in the corresponding Figure legends as suggested in the revised version. With regard to this putative structure in the main text, we mainly wanted to depict clearly to the readers what this hairpin looks like and to illustrate how the hairpin disruption is generated, thus placing this in the primary figures may assist the readers to understand this structure and the mechanism by which it influences PTENε expression more intuitively and vividly.

Q1.7. *In Figure 5A, the authors aim to specifically overexpress the PTEN-alpha isoform. However, as this mRNA also contains the starting codon for beta and epsilon, the question is whether they are overexpressing all the isoforms at the same time. Did the authors mutate the starting codon for beta and epsilon in order to specifically overexpress alpha? The same controls should be taken into consideration for PTEN-beta overexpression.*

R1.7. Thank you for your professional comments and detailed instructions and we feel apologetic about the ambiguous representation in the main text. Originally when we devised the C-GFP tagged PTEN isoforms constructs, we utilized the Kozak sequence and canonical initiation codon ATG to replace the alternative translation start sites of PTEN isoforms PTENα, PTENβ, and PTENε to enhance the expression of corresponding PTEN variants and alleviate the translation of other potential downstream alternative translation start sites. Moreover, to ensure each plasmid shown

in Fig 5A can only produce a single individual isoform, we performed western blotting in C-terminal GFP-tagged PTENα, PTENβ, PTENε, and PTEN overexpressed Hela *PTEN*$^{-/-}$ cells as suggested and found that there is indeed only one single isoform expressed in each instance (Appendix Fig S3a).



Appendix Fig S3a. Determination of the expression of C-terminal GFP tagged PTEN variants.

Q1.8. *The authors present an N-terminus folding prediction for PTEN-epsilon. Do the PTEN-alpha and beta isoforms that contain longer N-terminals fold differently?*

R1.8. Accordingly, SWISS-MODEL was used to predict the structure of PTENα, PTENβ by analyzing respect N-terminal extended amino acid sequence compared with canonical PTEN. As shown below, PTENα and PTENβ isoforms possess a different N-terminal structure compared with the PTENε variant. The number shown below represents the amino acid sites of corresponding PTEN isoforms.

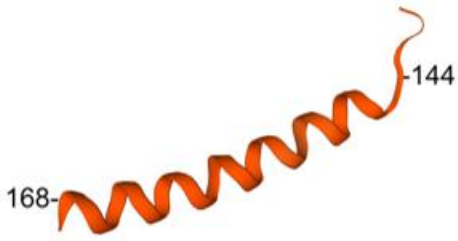


Fig R1.2. The putative N-terminal structure of PTENα predicted by SWISS-MODEL.

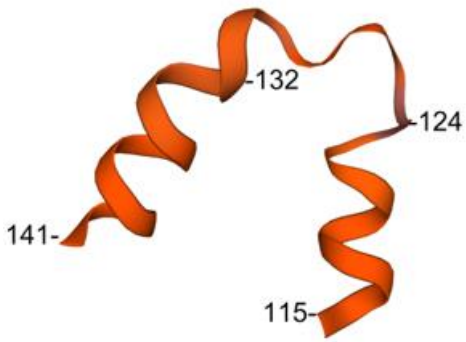


Fig R1.3. The putative N-terminal structure of PTENβ predicted by SWISS-MODEL.

Q1.9. *It is interesting that the authors show in Figure 7E-F that deleting only the PTEN-*

epsilon can increase cell motility. However, to strengthen the claim on the functional activity for this specific PTEN isoform, can the authors also include additional controls, such as overexpression of the other PTEN isoforms?

R1.9. We are grateful for the reviewer's helpful comments and detailed instructions. To address this issue, we established PTEN variants stable transfectants by infecting the Hela *PTEN*$^{-/-}$ cells with lentiviral-PTEN, lentiviral-PTENα, lentiviral-PTENβ, or lentiviral-PTENε respectively. Subsequently, the motility of stable transfectants was determined by transwell migration and matrigel invasion assays. Compared with the control cells, cell migration and invasion abilities of PTENα, PTENβ, PTENε or canonical PTEN overexpressed Hela cells were all reduced, while PTENε and PTENα have a stronger inhibitory effect on tumor invasion and metastasis than PTENβ and the canonical PTEN. This indicates that PTEN isoforms are involved in the inhibition of tumor invasion and metastasis through various mechanisms. As discussed in the main text, the role of canonical PTEN protein in suppression of metastasis has been studied extensively (Furukawa *et al*, 2006; Tamura *et al*, 1998). In the current study, we demonstrate that PTENε suppresses tumor metastasis through filopodia formation regulation. However, the mechanism of PTENα and PTENβ in modulating cell motility is still vague and needs further investigation.

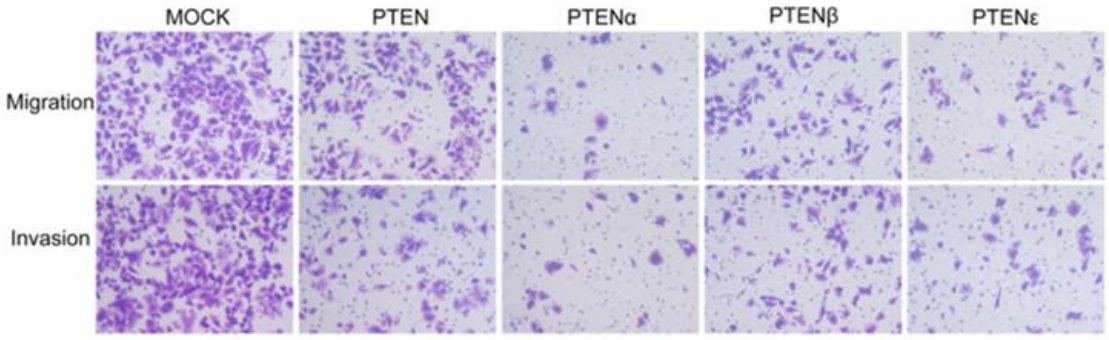


Fig R1.4. Representative images of transwell migration and matrigel invasion assays in PTEN variants overexpressed Hela cells.

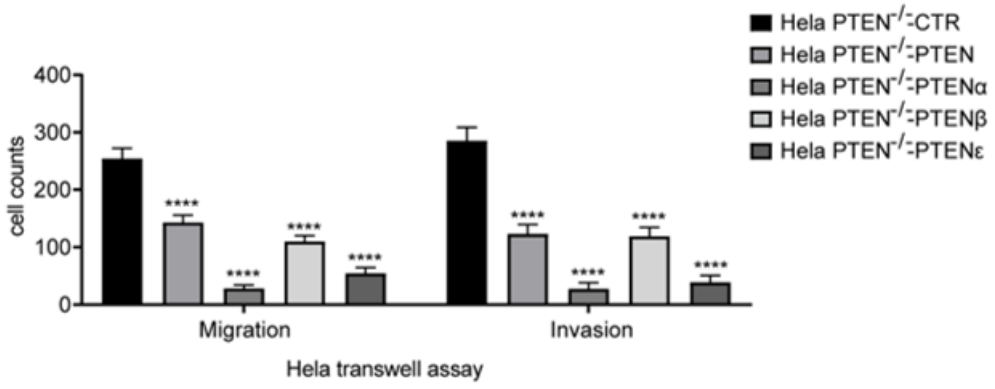


Fig R1.5. Statistical analysis of transwell migration and matrigel invasion assays in PTEN variants overexpressed Hela cells.

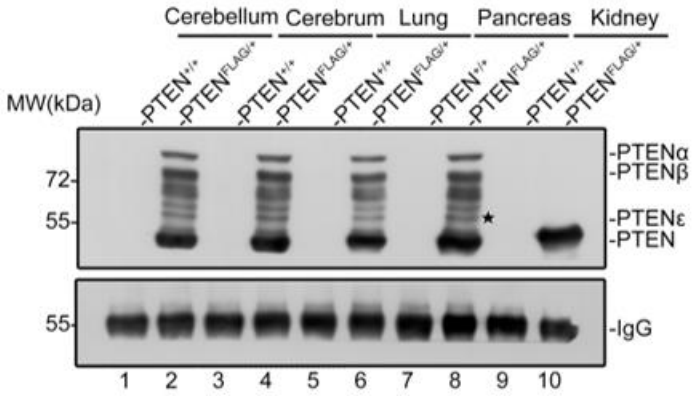
B. Point-by-point responses to the comments by Reviewer #2

In this manuscript, Yin's group identified a novel N-terminal-extended PTEN isoform designated as PTENε, of which the translation is initiated from the CUG^{816} codon within the 5'UTR region of PTEN mRNA. They also revealed that PTENε mainly localizes in the cell membrane, and dephosphorylates VASP and ACTR2. Overexpression of PTENε reduces filopodia formation and inhibits the metastasis of tumor cells. These original findings are interesting and suitable to be published in EMBO J, but some concerns should be addressed.

We appreciate the reviewer's favorable opinions and constructive comments. In the new revision, more information about the correlation between PTENε and eIF2A and the effect of membrane localization of PTENε on filopodia formation have been added and reorganized as suggested. Some new data have also been added to better support the regulation of PTENε on filopodia formation by dephosphorylation of ACTR2 and VASP. We have addressed each comment, and details are listed as follows.

Q2.1. *In figure 3D, they should also blot these precipitates with anti-PTEN antibody which show canonical PTEN besides its long isoforms.*

R2.1. Accordingly, the tissue samples extracted from heterozygous *Pten^{FLAG}* mice mentioned in Fig 3D were immunoblotted with an anti-PTEN antibody (Cell Signaling Technology, 138G6). As shown in Fig EV1B and 1C, we calculated the relative expression of PTENε compared with canonical PTEN in different tissues by evaluating the grayscale of relative protein bands and found that the relative expression of PTENε is higher in the cerebellum and cerebrum, suggesting that it may play specific roles in the nervous system as F-actin and filopodia-like structures are critical for the normal function of neurons.



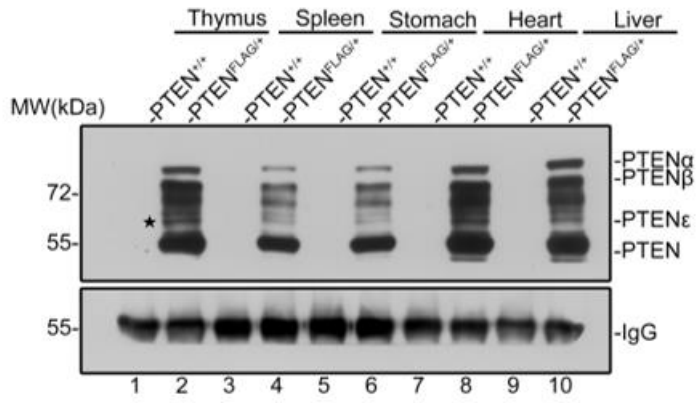


Fig EV1B. Verification of the expression of FLAG-tagged PTEN and PTEN isoforms in *Pten*^{FLAG} knock-in mice by an anti-PTEN antibody. The asterisk shown here and in other immunoblot images throughout this manuscript indicates the corresponding protein band of PTENε.

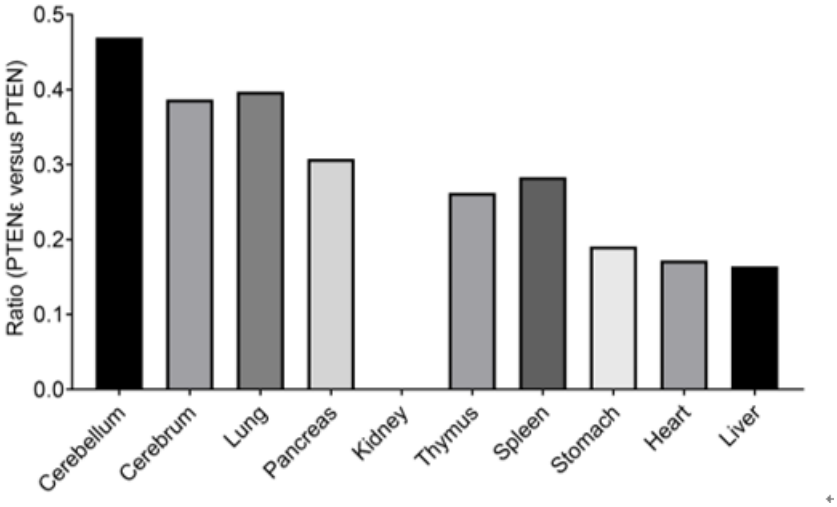


Fig EV1C. The relative tissue distribution of PTENε compared with canonical PTEN.

Q2.2. *They described that the level of eIF2A transcripts and protein positively correlated with PTENε protein in Figure 4A, but they actually only detected transcripts rather than proteins of two kinds of eIFs. I propose that they should also compare with correlation of their proteins.*

R2.2 We thank the reviewer for the careful review of our data and we agree that the statement mentioned is inaccurate. In light of the reviewer's suggestion, we detected the endogenous protein level of eIF2A in cell lines used in Fig 1B and calculated the protein level correlation between PTENε and eIF2A. We found that the protein level of eIF2A is positively correlated with PTENε expression, suggesting that eIF2A is related to the efficiency of PTENε alternative initiation (Fig EV2A).

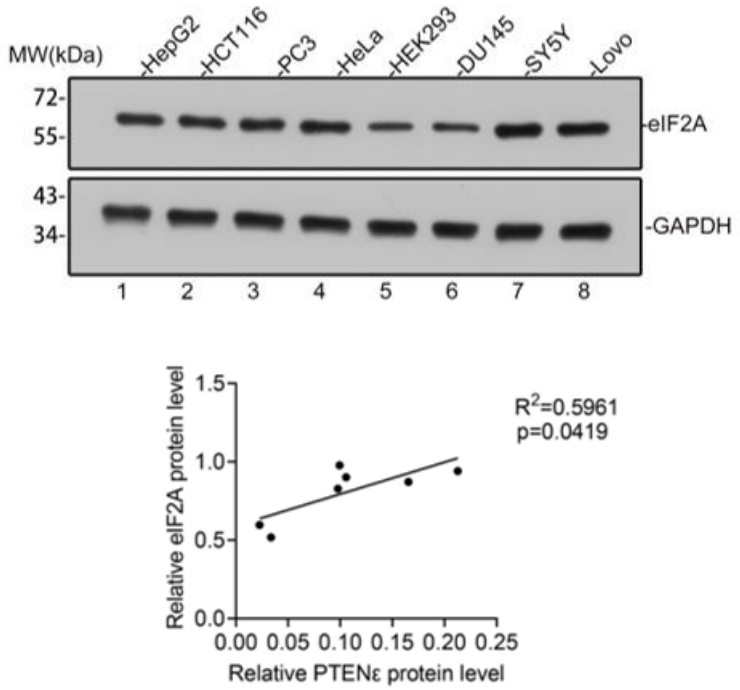


Fig EV2A. eIF2A protein level is positively correlated with endogenous expression of PTENε.

Q2.3. *Whether does endogenous PTENε inhibit phosphorylation of VASP and ACTR2?*

a) *To see the endogenous role of PTENε, they should detect the levels of VASP and ACTR2 phosphorylation in PTENε knockout cells.*
b) *They should also use phosphatase-deficient mutants as negative controls in Figure 4H/I, and use PTEN and other isoforms as negative controls in Figure 4G.*
c) *Also, they should point out that overexpression or knockdown of eIF2A also significantly regulated other several long isoforms of PTEN such as PTENα/β besides PTENε expression in Figure 4C/D, while disruption of the palindromic sequence leads to a marked reduction of PTENε rather than PTENα/β expression in Figure 4F. Did these results indicate the same mechanisms to regulated expressions of these PTEN variants?*

R2.3. We are very thankful for the reviewer's professional instructions. The following experiments were carried out to address these concerns.

a) The levels of endogenous phosphorylation of VASP and ACTR2 in Hela PTENε$^{+/-}$ cells were evaluated by western blotting using respective antibodies. As was shown in the revised Fig EV4C, the endogenous phosphorylation of VASP Ser157 and ACTR2 Thr237 and Thr238 sites was elevated in Hela PTENε$^{+/-}$ cells compared to Hela WT cells，supporting our conclusion that PTENε acts as VASP and ACTR2 phosphatase in vivo.

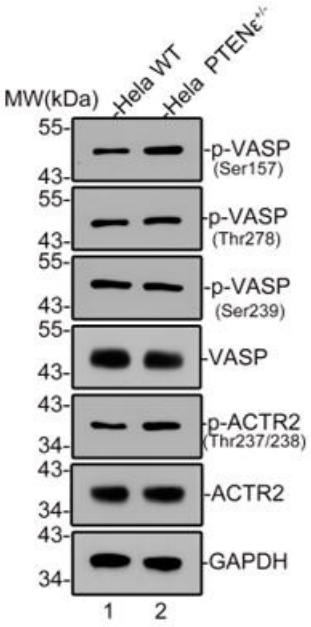


Fig EV4C. Endogenous PTENε depletion upregulates the phosphorylation of VASP and ACTR2.

b) We agree that phosphatase-deficient mutants should be included as negative controls to support our statement that PTENε may suppress filopodia formation through its protein phosphatase activity. Accordingly, in vitro dephosphorylation assays were carried out. His-PTENε, His-PTENε Y210L, His-PKA, and His-NIK were expressed in Sf9 cells and purified using Ni-NTA agarose (Qiagen). FLAG-VASP and FLAG-ACTR2 were purified with ANTI-FLAG® M2 Affinity Gel (F2426) and 3×FLAG Peptide (F4799) from HEK293 cells after transfection with pCMV-VASP and pCMV-ACTR2 for 30h. For kinase assay, purified FLAG-VASP or FLAG-ACTR2 on anti-FLAG M2 magnetic beads was incubated with His-PKA or His-NIK separately in kinase buffer for 30min at 30℃. During the following phosphatase assay, phosphorylated FLAG-VASP or FLAG-ACTR2 was incubated with purified His-PTENε or His-PTENε Y210L in dephosphorylation buffer for 1h at 37℃. The phosphorylation levels of VASP and ACTR2 sites with the potential for alteration were evaluated by western blotting. We found that the phosphatase-deficient mutant PTENε Y210L is unable to dephosphorylate VASP ser157 or ACTR2 Thr237/238 sites in comparison with wild-type PTENε (Revised Fig EV3D), which further verifies that PTENε indeed is able to dephosphorylate VASP and ACTR2 through its protein phosphatase activity.

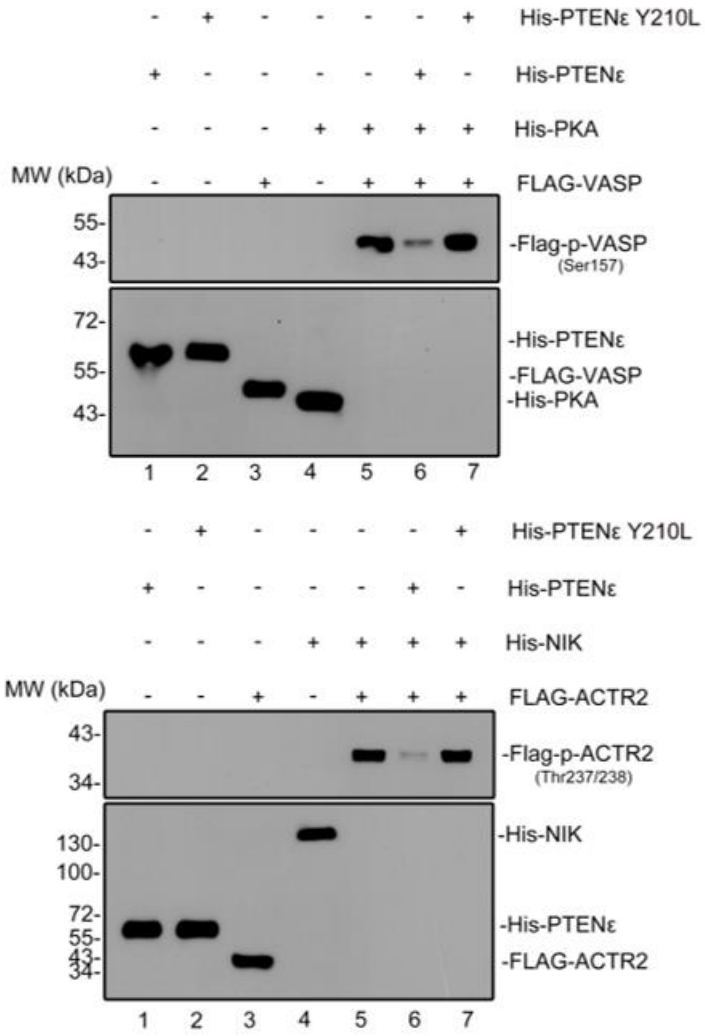


Fig EV3D. In vitro dephosphorylation assays to evaluate the protein phosphatase activity of PTENε on ACTR2 and VASP.

As suggested, the influence of other known PTEN variants on the phosphorylation of VASP and ACTR2 at corresponding sites was evaluated in these PTEN variants overexpressing cells. As shown in Fig EV3C, neither overexpressed PTENα, PTENβ nor canonical PTEN influences the phosphorylation status of VASP at Ser157 or ACTR2 at Thr237 and Thr238 sites, suggesting that PTENε but not other known PTEN variants, acts as the phosphatase of endogenous VASP and ACTR2.

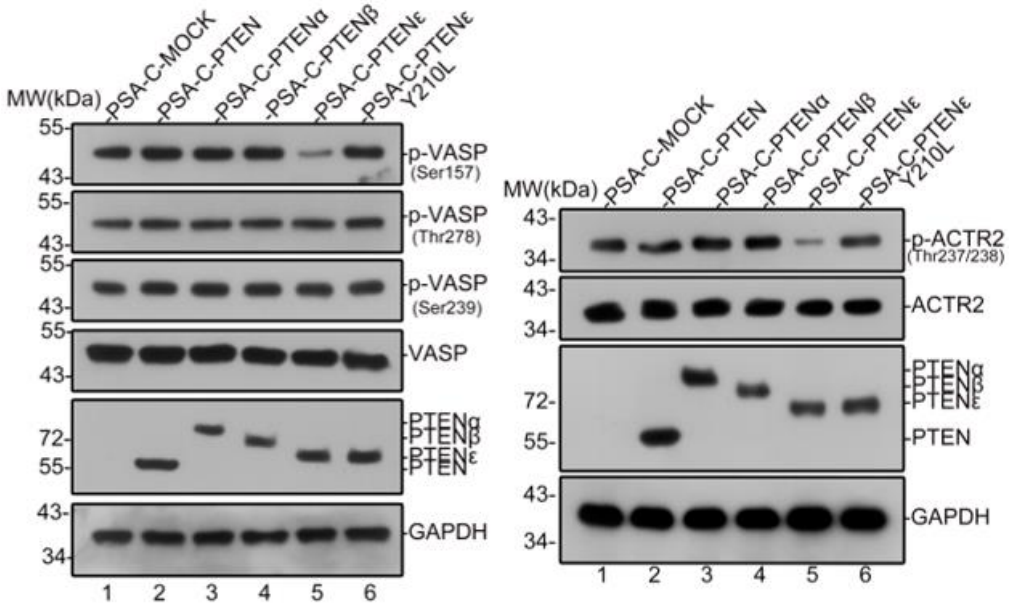
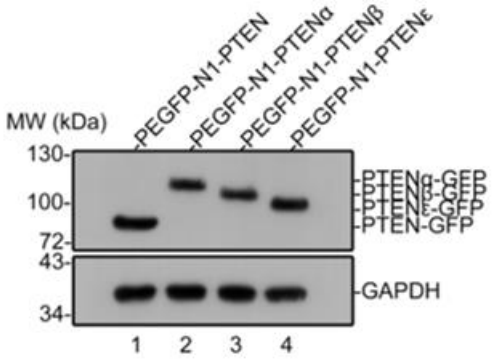


Fig EV3C. PTENε, but not other PTEN variants, reduces the phosphorylation of endogenous VASP and ACTR2.

c) We have readdressed the corresponding content in our manuscript according to your suggestion and the influence of eIF2A alteration on the expression of other PTEN isoforms has been added to the corresponding part of the manuscript. The ability of mammalian ribosomes to initiate at non-AUG codons is usually inefficient and can be facilitated by the flanking sequence and the presence of a stem-loop structure downstream of the alternative initiation sites (Diaz de Arce *et al*, 2018). As the previous research in our lab has demonstrated (Liang *et al*, 2017; Liang *et al*, 2014), there indeed exists similar hairpin around or downstream of PTENα or PTENβ alternative translation site, and the disruption of these hairpins selectively influenced the corresponding PTEN variants, which is consistent with the observation in Fig 4F, suggesting the putative hairpin may act as a formidable element in regulating the translation efficiency of specific non-AUG codons.

Q2.4. *Fig. 5A should show western blot to ensure expression of only a single isoform in each instance.*

R2.4. As suggested, western blotting in C-terminal GFP-tagged PTENα, PTENβ, PTENε, and PTEN overexpressed Hela *PTEN*$^{-/-}$ cells was performed and it shows that only one single isoform is expressed in each instance (Revised Appendix Fig S3a).



Appendix Fig S3a. Determination of the expression of C-terminal GFP tagged PTEN variants.

Q2.5. *It is hard to understand how PTENε showed different cellular localization from PTENα and PTENβ. It also contains nucleus-localized signal recently identified in PTENα/β. Actually, according to Figure 5B-D, PTENε appears be seen in cytoplasm and nucleus besides on the cell membrane. I also like to know whether PTENε is localized into nuclei by fractionation analysis in Figure 5D. By the way, the cervical cancer cell line Hela is used throughout the whole study. However, according to this reviewer's knowledge, Hela cells do not express E-cadherin (PMID: 12751384; PMID: 7636625). Why did authors detect strong E-cadherin signal in their Hela cells in Figure 5D/G.*

R2.5. We thank the reviewer for these insightful questions and careful review of our data.

a) In light of the reviewer's suggestion, to test whether PTENε may also localize into nuclei, we utilized HEK293 cells transfected with C-terminal GFP tagged PTENε to perform the nuclear and cytoplasmic protein extraction experiment. As was shown in Fig R2.1, only a small fraction of PTENε was detected in the nucleus.

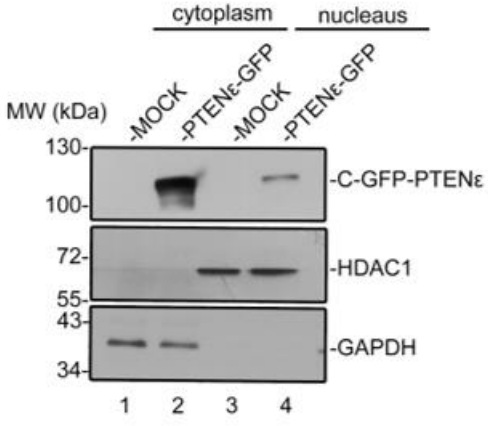


Fig R2.1. PTENε can also localize into nucleus like known isoforms PTENα and PTENβ.

Several reasons may account for the different cellular distribution of PTENε compared with PTENα and PTENβ. Firstly, the N-terminal sequences of these three isoforms are of different length, thus they may possess distinct conformations in comparison with each other. We also used the SWISS-MODEL webtool to predict

the structure of PTENα, PTENβ by analyzing respect N-terminal extended amino acid sequence compared with canonical PTEN. As was shown below, PTENα and PTENβ isoforms may indeed possess a different N-terminal structure compared with the PTENε variant. Secondly, according to the pulldown assay mentioned in the manuscript Fig 6A and the previous research by us (Liang *et al.*, 2014) and other groups, these PTEN variants can interact with proteins with distinct subcellular distribution, which may partially contribute to the various distribution of PTEN isoforms.

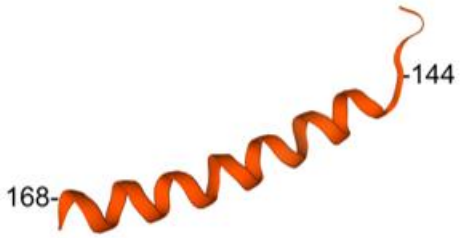


Fig R2.2. The putative N-terminal structure of PTENα predicted by SWISS-MODEL. The number shown above represents the amino acid sites of corresponding PTEN isoforms.

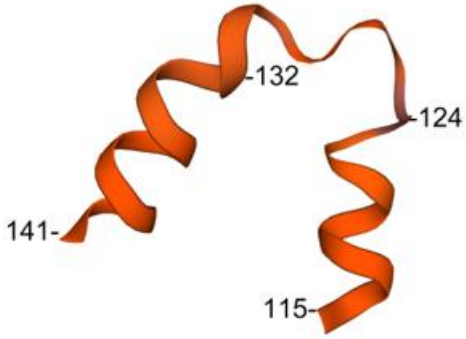


Fig R2.3. The putative N-terminal structure of PTENβ predicted by SWISS-MODEL. The number shown above represents the amino acid sites of corresponding PTEN isoforms.

b) We apologize for the reviewer's confusion caused by this mislabeling. We actually did the immunofluorescence using Hela cells and the cell fractionation using HEK293 cells. We have carefully checked the entire manuscript to avoid such ridiculous mistakes and substitute the mislabeling with correct statements in the revised manuscript.

Q2.6. *PTENα/β also own the amino acid sequence of PTENε protein. They should also address whether PTENα/β interacted with VASP, FSCN1, CDC42 and ACTR2 to support their conclusion for the specificity of the interaction of PTENε with these membrane proteins.*

R2.6. Accordingly, the interaction between PTENα/β and VASP, FSCN1, CDC42, and ACTR2 was tested by the co-IP experiment. As was shown in revised Fig EV3A, C-terminal HA-tagged PTEN variants (PTENα, PTENβ, PTENε, and canonical PTEN) in

the HEK293 cells were overexpressed separately and the co-IP experiment was subsequently performed. We found that only the C-terminal HA-tagged PTENε could physically associate with endogenous VASP, FSCN1, CDC42, and ACTR2 while canonical PTEN and the other two PTEN isoforms (PTENα and PTENβ) couldn't, which supports our conclusion that these molecules are specific interactors of PTENε.

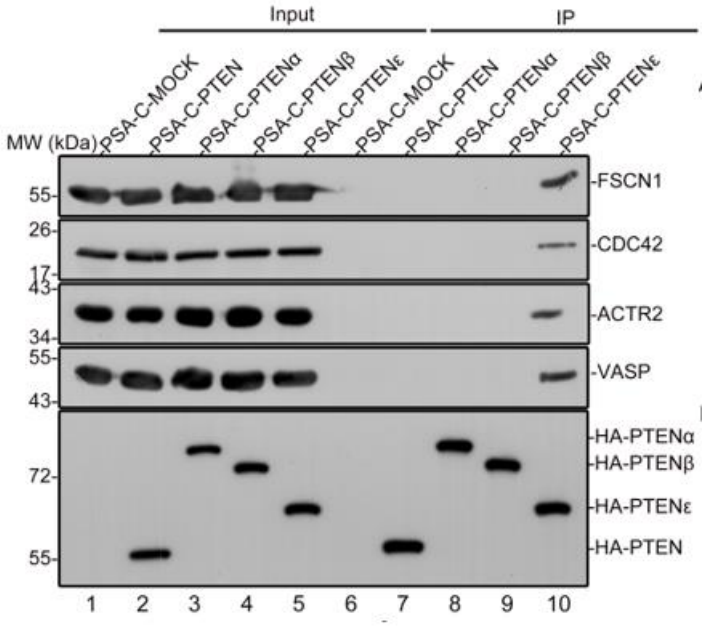


Fig EV3A. PTENε, but not other known PTEN isoforms, interacts with filopodia formation related proteins.

Q2.7. *The authors showed that PTENε played a role in filopodia formation, migration and invasion.*

a) they did not test the involvement of plasma membrane localization or VASP and ACTR2 in these processes.

b) Considering the small amount of PTENε even compared to PTENα and PTENβ, the endogenous role of PTENε should be tested in these processes within the same cell line, for example, B16 cells used for the mouse pulmonary metastasis model.

R2.7. We thank the reviewer for this comment.

a) We agree that the involvement of PTENε plasma membrane localization on filopodia formation should be done to strengthen our statement about the role PTENε played in filopodia formation, migration, and invasion. As suggested, C-terminal GFP tagged PTENε or the plasma membrane localization abolished PTENε mutant (PTENε 69-81aa delete) or mock construct was introduced into Hela *PTEN*$^{-/-}$ cells separately. The transfected cells were stained with Phalloidin and DAPI before being imaged with confocal microscopy. More than 30 cells with fluorescence signals were checked in each group. As was shown in the revised manuscript Fig EV4A and 4B, the overexpression of PTENε reduces the number of filopodia in the cell membrane while the membrane localization abolishment mutant shows no effect on filopodia formation compared with the mock group, suggesting that the membrane localization of PTENε indeed participates in the

function of PTENε on manipulating filopodia formation.

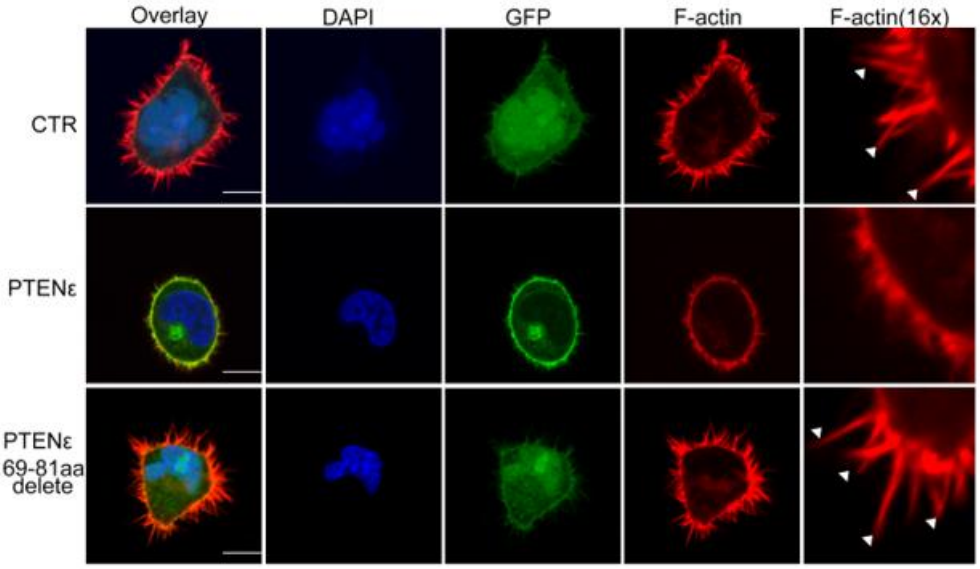


Fig EV4A. Influence of PTENε membrane localization on filopodia formation.

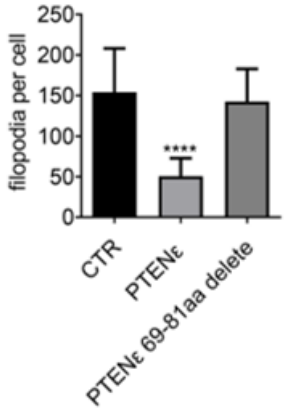


Fig EV4B. Statistical analysis of the influence of PTENε membrane localization on filopodia formation.

As to assess the involvement of VASP and ACTR2 in PTENε mediated filopodia formation regulation, we first detected the endogenous phosphorylation of VASP and ACTR2 at Ser157 and Thr237/238 sites respectively on Hela PTENε$^{+/-}$ cells and found that the depletion of endogenous PTENε induces the phosphorylation of these two proteins at corresponding sites (Revised Fig EV4C). Meanwhile, in vitro dephosphorylation assays further verified that PKA-phosphorylated VASP Ser157 and NIK-phosphorylated ACTR2 Thr237 and Thr238 sites are targets of PTENε for dephosphorylation by its protein phosphatase activity (Revised Fig EV3D). Based on these results and the fact that the phosphorylation of VASP ser157 and ACTR2 Thr237/238 sites is important for filopodia formation, we thus suspect that PTENε may suppress filopodia formation partially by dephosphorylating VASP and ACTR2, meanwhile, other molecules such as FSCN1 and CDC42 may also involve in this process through a mechanism that remains further validation.

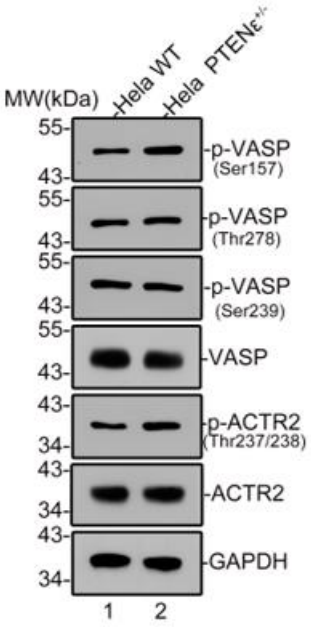
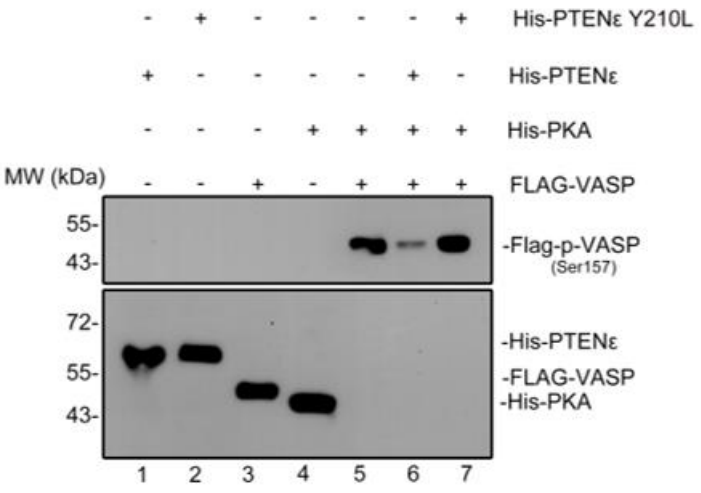


Fig EV4C. Endogenous PTENε depletion upregulates the phosphorylation of VASP and ACTR2.



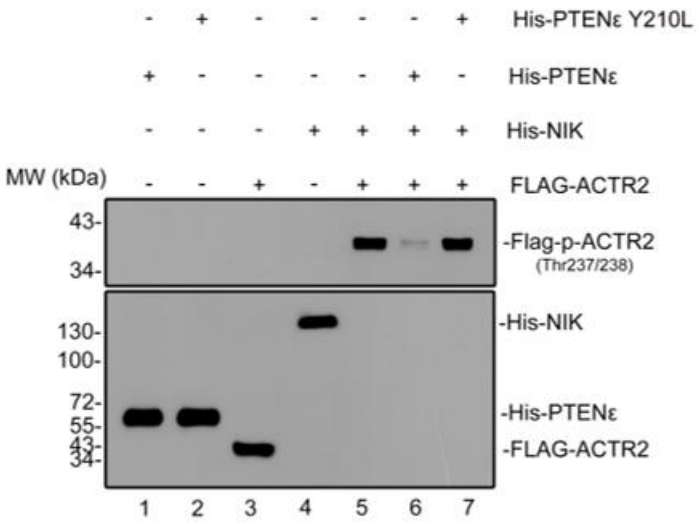


Fig EV3D. In vitro dephosphorylation assays to evaluate the protein phosphatase activity of PTENε on ACTR2 and VASP.

b) To testify whether the endogenous depletion of PTENε could influence the status of filopodia, we analyzed the fluorescence signals of more than 30 cells in Hela WT cells and Hela PTENε$^{+/-}$ group respectively. As was shown in the revised Fig EV4D and 4E, there are much more filopodia formed in Hela PTENε$^{+/-}$ cells compared with the parental Hela panel, further supporting our assumption that PTENε may participate in modulating filopodia formation.

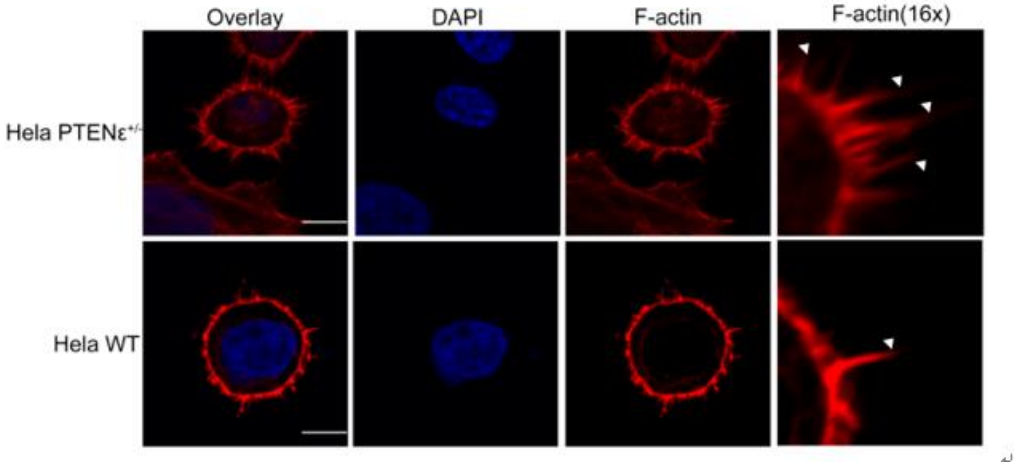


Fig EV4D. Endogenous PTENε depletion promotes filopodia formation in Hela cancer cells.

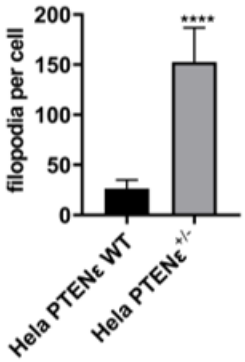


Fig EV4E. Statistical analysis of endogenous PTENε depletion on filopodia formation.

Minor concerns:

Q2.8. *In Figure 1A, they showed four unidentified proteins of molecular weights lower than PTENβ (between 72 and 55kDa) in Hela cells, which are designated as γ, δ, ε, and ζ isoforms of PTEN. I proposed that it is better to label all these isoforms in all 4 panels of Figure 1.*

R2.8. We apologize for the reviewer's confusion caused by our inaccurate statement.

In Figure 1A, four proteins of molecular weights lower than PTENβ (between 72 and 55kDa) were identified in Hela cells. As shown in Fig 1A line 2 versus line 1, the insertion of a TAG stop codon immediately downstream of the PTENβ initiation codon AUU^{594} only led to the disappearance of the slower migrating protein-forms PTENα and PTENβ without any effect on the protein bands slightly below PTENβ and above canonical PTEN, thus ruling out the possibility that some of these unidentified proteins are the cleavage products or modified versions of PTENα and PTENβ.

Besides, a TAG stop codon that terminates translation from upstream initiation sites inserted upstream of the AUG^{1032} start codon of canonical PTEN with CRISPR-Cas9 eliminated these bands slightly below PTENβ and above canonical PTEN without affecting canonical PTEN expression (Fig 1D, lane 2 versus lane 1), excluding the possibility of these unidentified proteins to be the bands of canonical PTEN modifications.

Furthermore, these protein bands slightly below PTENβ and above canonical PTEN can be efficiently recognized by the anti-PTENα antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENα (Fig 1C), indicating that they were more likely to be novel N-terminally extended PTEN variants.

In the current study, the existence of PTENε (or PTEN5) was verified through mutational analysis and mass spectrometry, while the translation initiation of the other three proteins with molecular weights slightly below PTENβ and above canonical PTEN was still undetermined, which will our future direction. Considering that these new isoforms are putative and not characterized, thus it is improper for them to be definitely designated as γ, δ, and ζ isoforms of PTEN in the current work. In the revised version "We proposed unified nomenclature for PTEN proteo-forms where newly

identified PTEN isoforms are designated as γ, δ, ε, and ζ isoforms of PTEN or PTEN3, PTEN4 PTEN5 and PTEN6 by the order of molecular weight of proteins, following PTENα and PTENβ." has been changed to "By the order of molecular weights of proteins following PTENα, the PTEN proteo-form ranked fifth is designated as PTENε or PTEN5.". The band of PTENε in immunoblot images has been labeled with an asterisk in order to distinguish it from other unidentified proteins with similar molecular weights.

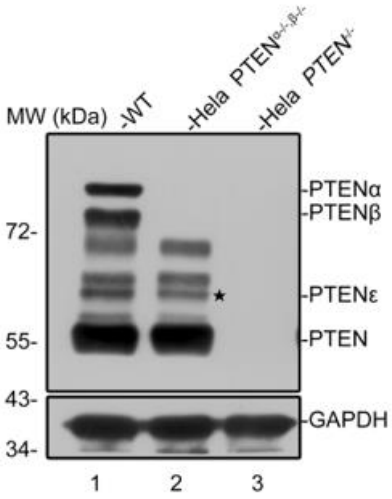


Fig 1A. The discovery of novel PTEN variants in Hela PTEN$^{\alpha-/-,\beta-/-}$ cells.

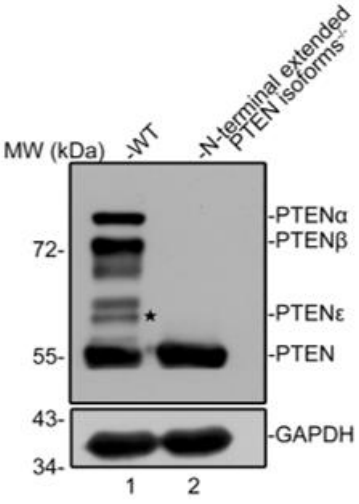


Fig 1D. Disruption of the PTEN mRNA 5'UTR eliminates expression of all PTEN isoforms.

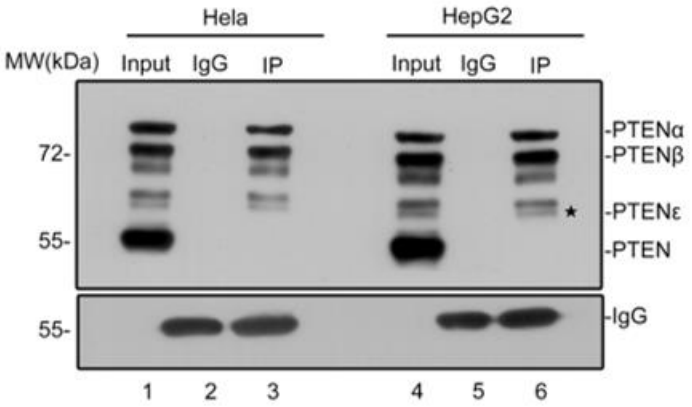


Fig 1C. The endogenous immunoprecipitation in the Hela and HepG2 cancer cell lines by the anti-PTENα antibody.

Q2.9. *There are too many mistakes when describing their results and mis-spelling. For examples, legend descriptions are inconsistent with some Figure, such as Figure 4B, 6D. When describing Figure 2F (page 7), lane 5 but not lane 4 should be compared to other lanes; In page 11, a serious of should be a series of; On page 8, "as listed in Fig. 1C." should be Fig. 1B. On page 12, subtract should be substrate; in Figure 5D/G, tululin should be tubulin. "we first examined the expression of eIF5 and eIF2A in a panel of cancer cell lines as listed in Fig. 1C.", a panel of cancer cell is listed in Fig. 1B, not Fig. 1C.*

R2.9. We are grateful for your careful review of our manuscript. We have carefully scrutinized the whole body of our manuscript and replaced all the mistakes and misspellings with corresponding accurate statements in the revised version.

Q2.10. *In the introduction, some novel reports on nuclear PTEN should be mentioned and cited such as Nat Commun.2018;9(1):2392;2020;11(1):1720;*

R2.10. According to the reviewer's comments, the explicit new and classical discoveries about the biological function of nuclear-localized PTEN has been expanded in the revised version.

C. Point-by-point responses to the comments by Reviewer #3

In this manuscript, authors identified PTENε (or PTEN5), as a novel N-terminal-extended PTEN isoform that suppresses tumor invasion and metastasis. They showed that PTENε mainly localizes in the cell membrane and affects filopodia formation and cell motility potentially through VASP and ACTR2 dephosphorylation. They found that its overexpression reduces filopodia formation and inhibits the metastasis capacity of tumor cells.

The concepts raised in the manuscript are interesting and constitute an advance in the understanding of the relevance and diversity of PTEN functions. Authors identified a novel isoform of PTEN with distinct localization and function compared to the known

members of the family. Overall, this is a very interesting and timely study for EMBO J. There are general comments and some suggestions that the authors could consider before publication.

We appreciate the reviewer's positive comments and instructions for improving the manuscript. In the revised version, more sequencing results about Hela PTENε$^{+/-}$ cells have been added to exclude the possible existence of any potential off-targets. Meanwhile, new data about the influence of PTENε on filopodia formation and cell morphology have been supplemented in the manuscript to support our statement that PTENε suppresses tumor metastasis through filopodia formation regulation. We have performed an additional series of experiments and addressed all the comments as below:

General comments:

Q3.1. *The biochemical evidence of the existence and function of PTENε is convincing. I have no major criticisms with the data presented and the conclusions reached.*

R3.1. Thank you for your positive comments and suggestions to improve our manuscript.

Q3.2. *The regulation of PTENε by eIF2 is intriguing. This protein is profoundly influence by post-translational modifications. It would be interesting to ascertain whether Integrated Stress Responses leading to eIF2A S51 phosphorylation alters the production of this isoform.*

R3.2. The protein that modulates PTENε expression is the Eukaryotic initiation factor 2A (eIF2A) but not the Alpha Subunit of eIF2 (eIF2α). Although these two proteins are much similar in spelling, they are different proteins involved in distinct cellular processes. eIF2α is the α-subunit of eIF2 which is about 36kDa in molecular weight and contains the main target residue for eIF2 regulation (through phosphorylation), a serine at position 51 (Donnelly *et al*, 2013; Wek, 2018) whereas eIF2A refers to a single chain 65kDa protein that acts at some minor/alternative initiation events such as re-initiation, internal initiation, or non-AUG initiation, important for translational control of specific mRNAs (Komar *et al*, 2005; Starck *et al*, 2012; Zoll *et al*, 2002). As far as we know, no phosphorylation site of eIF2A that influence its function has been reported.

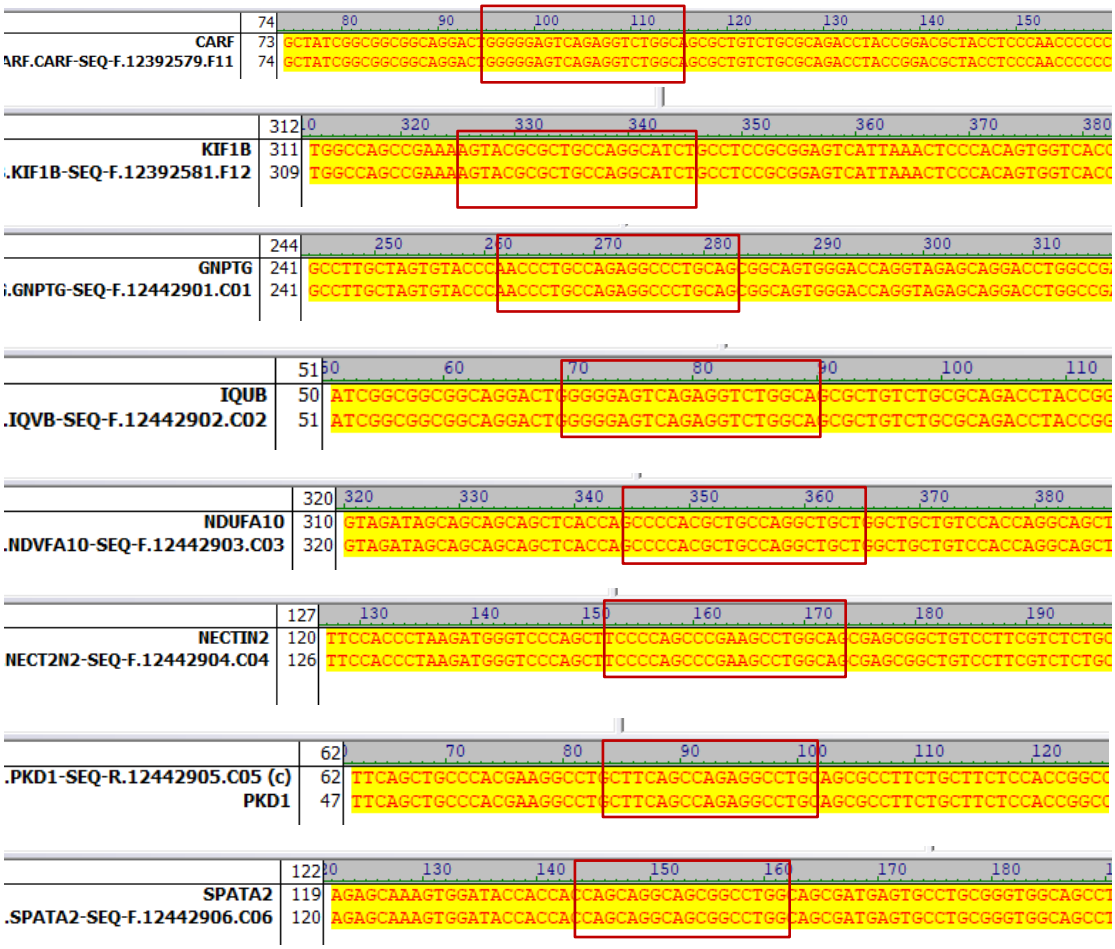
Q3.3. *CRISPR/Cas9 mutants: more information about genotyping approach should be provided, whether the lesion was homozygous/heterozygous, and whether any potential off-targets of the sgRNA were checked.*

R3.3. We agree that information about potential off-targets of the sgRNA should be provided. Accordingly, additional brief details about the establishment of PTENε$^{+/-}$ Hela cells have been added in the revised manuscript.

a) To obtain the endogenous PTENε depleted Hela cells, we utilized the Adenine Base Editing system to convert the alternative translation site CTG^{816} into CCG^{816} to

eliminate the translation of PTENε without affecting the translation efficiency of other PTEN variants. Technically, we synthesized the oligo (CACCGAGCCAGAGGCCTGGCAGCGG) from Tsingke and ligated them into a modified pX459 plasmid in which the cas9 sequence was deleted. Then Hela cells were transfected with this modified pX459 and ABE7.10 (purchased from Addgene) in a ratio of 1:3 according to the previously published paper by Nicole M. Gaudelli (Gaudelli *et al*, 2017). 24 hours after transfection, the positively transfected cells were selected by adding puromycin (2ug/ml) for 4 days. Then all the selected cell clones were amplified for DNA sequencing. The DNA sequencing result shown in Appendix Fig S7a reveals that we obtain a PTENε heterozygous knockout cell clone and the western blotting in Fig 7E shows endogenous expression of PTENε is indeed reduced in this heterozygous knockout cell clone.

b) To test whether there are any potential off-targets in our Hela PTENε$^{+/-}$ cells, we use the BLAST webtool in the NCBI portal to find out the transcripts that may be targeted by the sgRNA (AGCCAGAGGCCTGGCAGCGG). Then we selected the top ten matched transcripts except for PTEN for further analysis. Finally, DNA sequencing was used to verify whether the genome sequence of the corresponding misrecognized gene was altered in Hela PTENε$^{+/-}$ cells. As shown in Appendix Fig S7b, neither genome mutation nor deletion was detected in this heterozygous PTENε knockout cell clone, suggesting that there may not contain putative off-targets in Hela PTENε$^{+/-}$ cells.



Appendix Fig S7b. Detection of potential off-targets in Hela PTENε$^{+/-}$ cells. Red boxes indicate the mismatched regions in the corresponding gene.

Q3.4. *Some results (specifically clarified bellow) and discussion should be revised to avoid overstatements that go far beyond results. For instance, in the discussion authors state: "our data strongly indicate that PTENε exerts this function mainly through regulating the formation of pseudopods". However, results only indicate that filopodia formation is dependent on the phosphatase activity of PTENε (Figure 7a and b).*

R3.4. We agree that the statement referred to is inaccurate. Accordingly, in the new revision, the sentence "…our data strongly indicate that PTENε exerts this function mainly through regulating the formation of pseudopods…" has been changed to "…our data indicate that PTENε may exert this function through regulating the formation of pseudopods by its protein phosphatase activity…".

Q3.5. *A schematic representation of all the isoforms described would be important (some of the new isoforms are marginally mentioned, but represent an important finding). Also, these new isoforms should be indicated in the relevant panels of figure 1.*

R3.5. We apologize for the reviewer's confusion caused by our inaccurate statement. An explicit schematic explanation of isoforms translated from *PTEN* described in the manuscript is provided as suggested.

In Figure 1A, four proteins of molecular weights lower than PTENβ (between 72 and 55kDa) were identified in Hela cells. As shown in Fig 1A line 2 versus line 1, the insertion of a TAG stop codon immediately downstream of the PTENβ initiation codon AUU^{594} only led to the disappearance of the slower migrating protein-forms PTENα and PTENβ without any effect on the protein bands slightly below PTENβ and above canonical PTEN, thus ruling out the possibility that some of these unidentified proteins are the cleavage products or modified versions of PTENα and PTENβ.

Besides, a TAG stop codon that terminates translation from upstream initiation sites inserted upstream of the AUG^{1032} start codon of canonical PTEN with CRISPR-Cas9 eliminated these bands slightly below PTENβ and above PTEN without affecting canonical PTEN expression (Fig 1D, lane 2 versus lane 1), excluding the possibility of these unidentified proteins to be the bands of canonical PTEN modifications.

Furthermore, these protein bands slightly below PTENβ and above canonical PTEN can be efficiently recognized by the anti-PTENα antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENα (Fig 1C), indicating that they were more likely to be

novel N-terminally extended PTEN variants.

In the current study, the existence of PTENε (or PTEN5) was verified through mutational analysis and mass spectrometry, while the translation initiation of the other three proteins with molecular weights slightly below PTENβ and above canonical PTEN was still undetermined, which will our future direction. Considering that these new isoforms are putative and not characterized, thus it is improper for them to be definitely designated as γ, δ, and ζ isoforms of PTEN in the current work. In the revised version "We proposed unified nomenclature for PTEN proteo-forms where newly identified PTEN isoforms are designated as γ, δ, ε, and ζ isoforms of PTEN or PTEN3, PTEN4 PTEN5 and PTEN6 by the order of molecular weight of proteins, following PTENα and PTENβ." has been changed to "By the order of molecular weights of proteins following PTENα, the PTEN proteo-form ranked fifth is designated as PTENε or PTEN5.". The band of PTENε in immunoblot images has been labeled with an asterisk in order to distinguish it from other unidentified proteins with similar molecular weights.

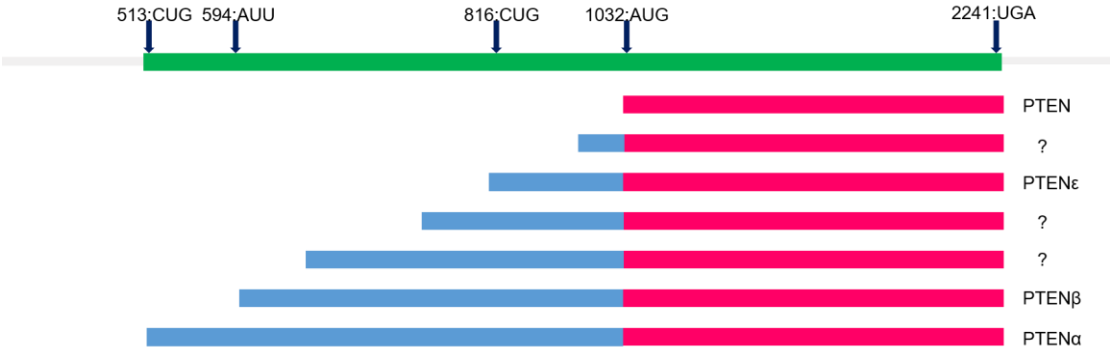


Fig R3.1. A schematic representation of PTEN family members. The label "?" stands for unidentified proteo-forms of PTEN that remain further verification.

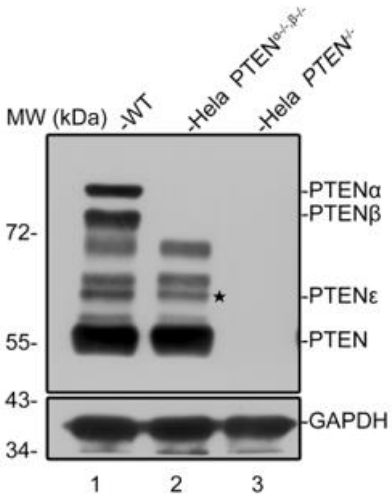


Fig 1A. The discovery of novel PTEN variants in Hela PTEN$^{\alpha-/-,\ \beta-/-}$ cells. The asterisk shown here and in other immunoblot images throughout this manuscript indicates the corresponding

protein band of PTENε.

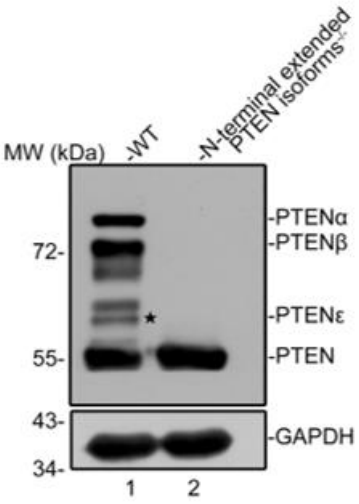


Fig 1D. Disruption of the PTEN mRNA 5'UTR eliminates expression of all PTEN isoforms.

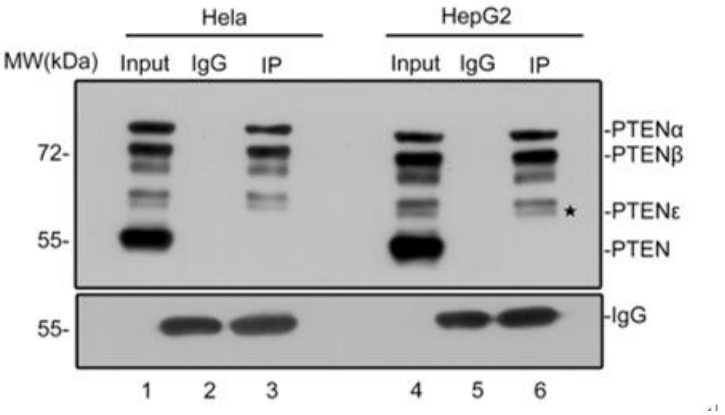


Fig 1C. The endogenous immunoprecipitation in the Hela and HepG2 cancer cell lines by the anti-PTENα antibody.

Q3.6. *The mass spectrometry-based determination is interesting. Although challenging, analyzing by mass spectrometry endogenous PTEN immunoprecipitants would be interesting.*

R3.6. As suggested, immunoprecipitation was performed in Hela cells with an anti-PTEN antibody (Cell Signaling Technology, 138G6), followed by SDS-PAGE and Coomassie Blue staining for protein detection. A protein band with a molecular weight lower than PTENα and PTENβ which is similar to the PTENε band identified in cancer cell lines was detected in Hela cells. This PTENε-like protein band was isolated and subject to protein sequence identification through mass spectrometry. As shown in Fig R3.2, mascot reports revealed 4 peptide fragments that cover 83.3% of the N-terminal region of PTENε and the most proximal N-terminal peptide of PTENε, MAAEEKQAQSLQPSSSR. Altogether, these results verify the existence of PTENε in vivo.

peptides	positions	sequences	modifications	q-Value	PEP	XCorr	Charge
1	1-17	mAAEEkQAQSLQPSSSR	M1(Oxidation)	0	8.59E-05	5.77	2
2	18-34	RSSHYPAAVQSQAAAER		0	4.57E-05	5.03	3
3	44-53	AISILQkkPR	K8(Propionyl)	0.001	2.13E-02	2.44	3
4	54-69	HQQLLPSLSSFFFSHR		0	4.531E-12	5.02	3

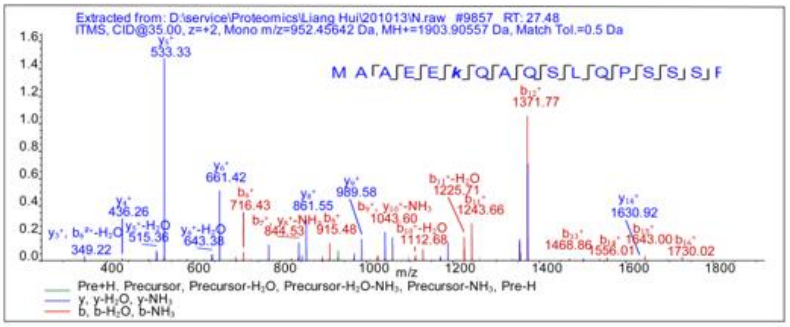


Fig R3.2. N-terminal sequencing of Hela endogenous PTENε enriched by an anti-PTEN antibody through the MS/MS spectrum.

Q3.7. *The filopodia differences in Fig. 7A and B are not obvious. Please, indicate what accounts for filopodia in magnification, and consider including more representative images.*

R3.7. In light of the reviewer's suggestion, we reperformed this experiment on PTENε overexpressed Hela cells, and representative images in magnification were supplemented in the revised version. Briefly, C-GFP tagged PTENε, PTENε Y210L (the protein phosphatase deficient mutant), and mock plasmid were introduced into Hela *PTEN*$^{-/-}$ cells separately. 24 hours after transfection, about $1*10^5$ cells were plated on coverslip coated with fibronectin (10ug/ml) at 4℃ overnight for further analysis. Then the transfected cells were stained with Phalloidin and DAPI before being imaged with confocal microscopy. As was shown below, overexpression of wild type PTENε in Hela *PTEN*$^{-/-}$ led to a sharp decrease of filopodia number, compared with control cells, while overexpression of PTENε Y210L had no such effect. The white arrows indicate the filopodia in the cell membrane and as was shown in the revised Fig 7B, the number of filopodia in the control panel and PTENε Y210L panel is much more than that in the PTENε overexpression group.

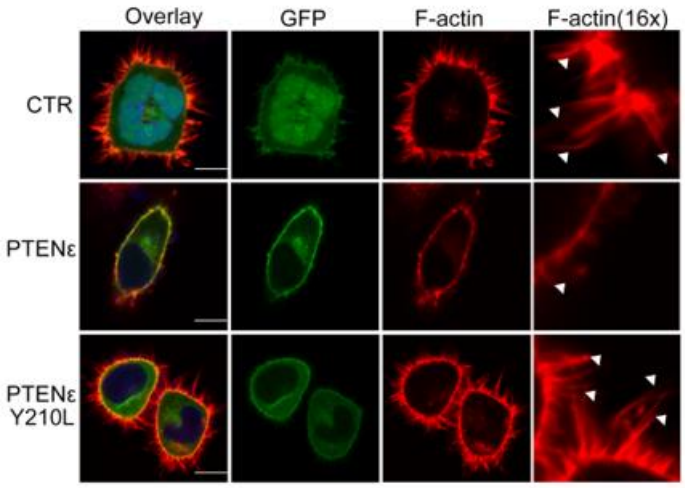


Fig 7A. The influence of PTENε overexpression on cell filopodia formation.

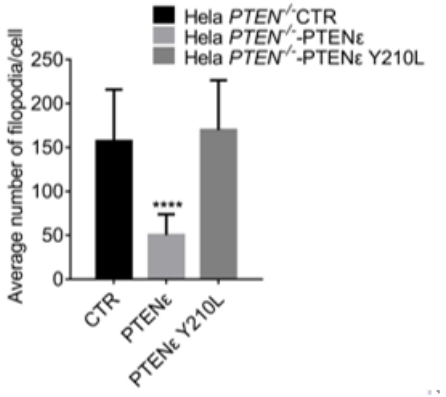


Fig 7B. Statistical analysis of the influence of PTENε overexpression on filopodia formation.

Q3.8. *In supplementary Fig. 8c, the differences in cell morphology is far from obvious.*

R3.8. Thank you for your knowledgeable advice in helping modify our manuscript. Given that there might be somewhat indirect when using exogenous expressed PTENε to explore the regulation of PTENε in filopodia formation and cell morphology, we thus reperformed this experiment in Hela PTENε$^{+/-}$ cells instead. As was shown in the revised Fig EV4F, Hela PTENε$^{+/-}$ cells were spindle-shaped, irregular and in a disordered arrangement group while the wildtype Hela cells were relatively single, short shuttle-like, or round shapes.

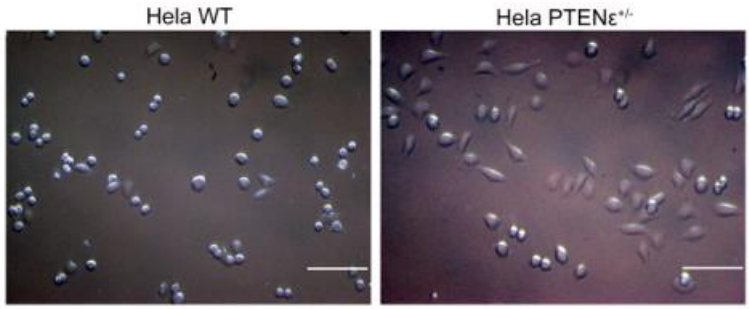


Fig EV4F. The influence of endogenous PTENε depletion on cell morphology.

Specific comments

Q3.9. *PTEN open-close conformation has also been associated to its activity and localization to membrane. How do the authors reconcile this?*

R3.9. Canonical PTEN localizes to cell membrane mainly through its N-terminal PIP2 binding motif and C2 domain. The interaction between PIP2 and PTEN N-terminal is modulated by the relative concentration of PIP2 versus PIP3 in the cell membrane (Redfern *et al*, 2008; Walker *et al*, 2004) while the membrane interacting residues within the C2 domain is normally masked by the C-tail domain due to C-tail phosphorylation by multiple kinases, only when the C-tail is dephosphorylated, did the C2 domain could interact with cell membrane thus converting PIP3 into PIP2 and suppressing PI3K/AKT pathway (Lee *et al*, 1999; Vazquez *et al*, 2000). However, although canonical PTEN and PTENε can both localize in the cell membrane, they interact with different partners as was shown in Fig 6B and C that canonical PTEN did not interact with these four proteins (VASP, ACTR2, CDC42, and FSCN1) that specifically interacts with PTENε. Therefore, we presume that membrane-localized PTENε and canonical PTEN may interact with different proteins thus executing different molecular functions.

Q3.10. *Figure 1a: Authors claimed that they ruled out the possibility that some of these unidentified proteins are cleavage products by performing a PTENα and PTENβ KO. However, at this point of the manuscript, they should mention that it is also possible that those lower MW bands could be modified forms of PTEN (ubiquitinated, phosphorylated...). Also, the authors depict PTENε as a double-band in the western blot. Can the authors discuss or prove the nature of these bands?*

R3.10. We thank the reviewer for the careful review of our data.

a) To rule out the possibility that those bands could be modified forms of PTEN, we introduced a TAG stop codon that terminates translation from upstream initiation sites in front of the AUG^{1032} start codon of canonical PTEN with CRISPR-Cas9 (Appendix Fig S1b), and this somatic mutation eliminated all the four PTEN-like proteins along with PTENα and PTENβ without affecting canonical PTEN

expression (Fig 1D, lane 2 versus lane 1), thus excluding the possibility of these unidentified proteins to be the bands of canonical PTEN modifications and indicating that these PTEN-like proteins were generated by initiation at an in-frame initiation codon within the PTEN mRNA 5'leader.

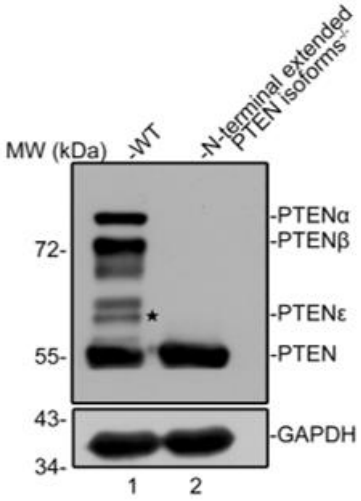


Fig 1D. Disruption of the PTEN mRNA 5'UTR eliminates the expression of all PTEN isoforms.

b) We feel very sorry for the misleading in depicting these unidentified bands. There may exist at least another four PTEN variants apart from PTENα and PTENβ. By the order of molecular weights of proteins following PTENα, the PTEN proteo-form ranked fifth is designated as PTENε or PTEN5, while the band slightly above or below PTENε was the other unidentified PTEN proteo-forms. The band of PTENε in immunoblot images has been labeled with an asterisk in order to distinguish it from other unidentified proteins with similar molecular weights.

Q3.11. *Figure 1b: why are different antibodies used in figure 1a and 1b (polyclonal antibody against full-length PTEN and rabbit monoclonal antibody against the C-terminal region of PTEN, Cell Signaling, 138G6, respectively)?*

R3.11. We feel very obliged for the reviewer's such a careful review of our manuscript. Using different antibodies that recognize different amino acids of canonical PTEN may be capable to mutually preclude the possibility that these recognized bands may be unspecific bands misrecognized by the antibody.

Q3.12. *Figure 1c: Using a PTENα antibody raised in the laboratory, authors identified three of the unidentified proteins with larger molecular weights than PTEN. Any idea about what the forth band on top of PTEN that cannot be identified using this antibody in figure 1c but that is present on figure 1a and b is?*

R3.12. We feel very grateful for the reviewer's careful review of our data and we understand the reviewer's concern. The fourth band on top of PTEN is an unidentified

PTEN variant, and the work on the identification and functional exploration of this PTEN variant is still in progress. PTENα polyclonal antibody was made by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENα. Considering the N-terminal sequence of this PTEN proteo-form (a new unidentified PTEN isoform according to our unpublished data) only encompasses a small amount of these 173 amino acids so it may be too short to be recognized by this self-made PTENα antibody.

Q3.13. *Figure 2a: It would be interesting to see the same western blot probed against PTEN antibody to detect and compare the endogenous levels of PTEN isoforms vs the overexpressed ones.*

R3.13. As suggested, the plasmid used in Fig 2A lane 3 was introduced into Hela *PTEN*$^{-/-}$ cells. 24 hours after transfection, the transfected cells and Hela WT cells were harvested and the lysates were immunoblotted with a PTEN monoclonal antibody (Cell Signaling Technology, 138G6). GAPDH was used as a control. Then the relative ratio of endogenous PTEN isoforms versus the overexpressed ones was analyzed by calculating the grayscale value in photoshop software. The relative ratio of endogenous isoforms versus overexpressed ones (PTENα, PTENβ, PTENε, and canonical PTEN) was shown below.

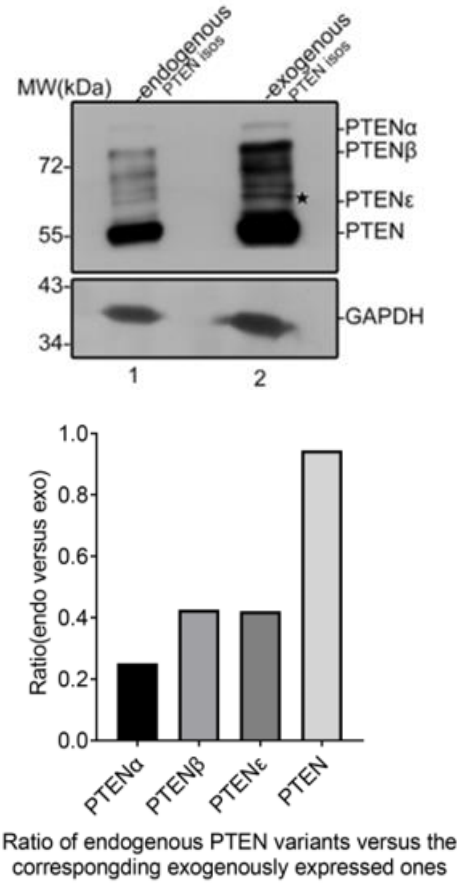


Fig R3.3. The ratio of endogenous PTEN isoforms versus the overexpressed ones used in Fig 2A lane3.

Q3.14. *Figure 3d: Don´t you expect to detect also canonical PTEN in the different tissues? Perhaps the ratio of these forms vs. the canonical PTEN isoform should be presented by blotting with a pan-PTEN antibody.*

R3.14. As suggested, we blotted the tissue samples extracted from heterozygous *Pten*^{FLAG} mice mentioned in Fig 3D with a monoclonal PTEN antibody (Cell Signaling Technology, 138G6). As suggested, we calculated the relative expression of PTENε compared with canonical PTEN in different tissues and found that the relative expression of PTENε is higher in the cerebellum and cerebrum which are all belong to the nervous system (Fig EV1B and 1C). Considering F-actin and filopodia-like structures such as dendrites and axons are crucial for the normal function of neurons, we suspect that PTENε may play an important role in the nervous system.

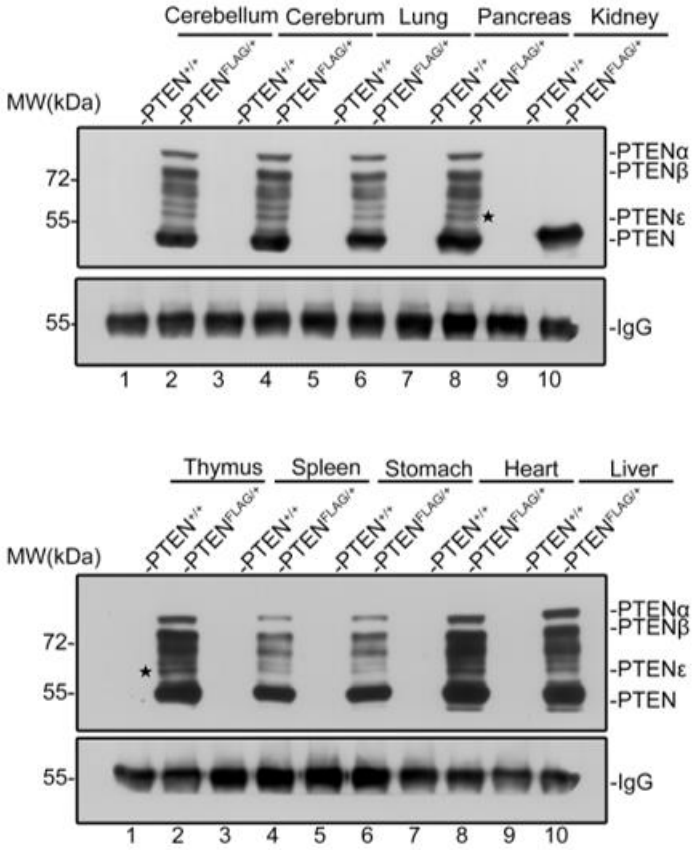


Fig EV1B. Verification of the expression of FLAG-tagged PTEN and PTEN isoforms in *Pten*^{FLAG} knock-in mice by an anti-PTEN antibody.

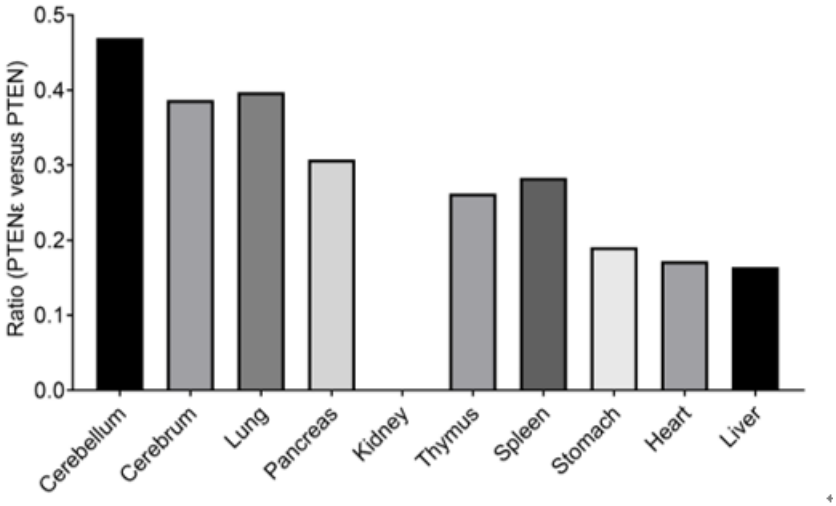


Fig EV1C. The relative tissue distribution of PTENε compared with canonical PTEN.

Q3.15. *Figure 4b: here, genetic rescue using reintroduction of FLAG-eIF2A and checking whether you rescue PTEN levels would be useful, although not essential.*

R3.15. We are grateful for the reviewer's constructive comment. Accordingly, the following experiments were carried out to address the comment.

(a) As suggested, genetic rescue by reintroduction of FLAG-eIF2A into eIF2A knockdown cells was performed. As PTENε expression is downregulated markedly in eIF2A knockdown cells, in the revised version, we further measured the level of PTEN isoforms in eIF2A knockdown Hela cells transfected with FLAG-eIF2A and it turns out that eIF2A overexpression compensates for the downregulation of PTENε and other N-terminal extended PTEN isoforms resulting from knockdown of endogenous eIF2A.

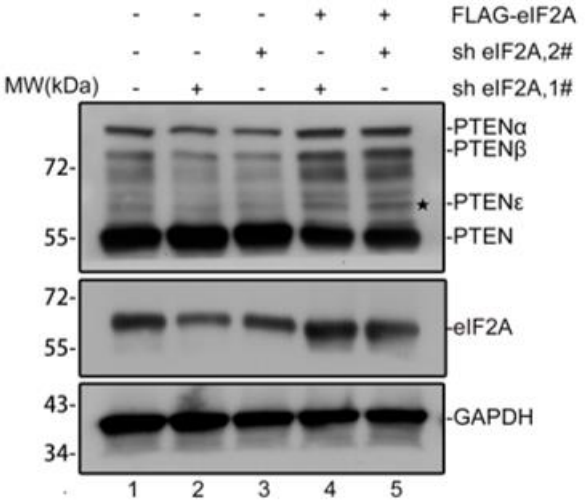


Fig EV2B. The reduced expression of PTEN isoforms in eIF2A knockdown cells was rescued by eIF2A reintroduction.

(b) We linked the 5'UTR of PTEN with a luciferase reporter to testify the influence of eIF2A alteration on the translation of distinct isoforms initiated by specific

upstream codons. Meanwhile, the non-AUG start codons that initiate other potential PTEN variants within the 5'UTR were mutated to CTC codons to ensure that only one isoform was expressed in each panel. Luciferase activity was measured with the Dual-Luciferase Assay System (Promega) following the manufacturer's protocol. We found that overexpression of eIF2A indeed promotes the translation of PTENα, PTENβ, and PTENε, respectively (Fig EV2D).

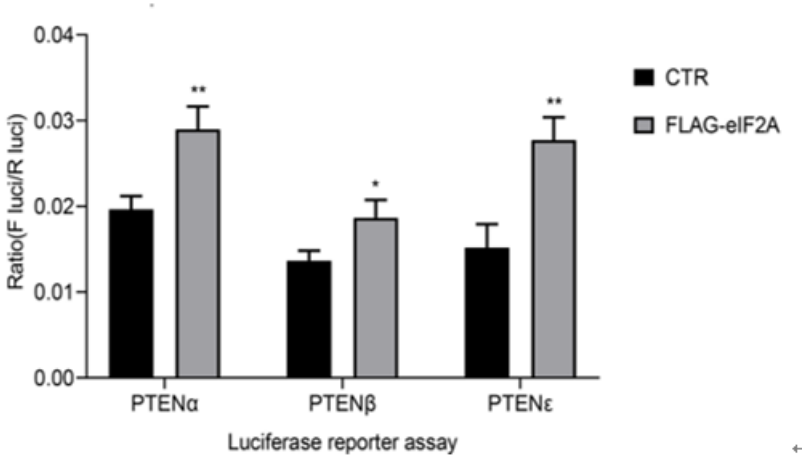


Fig EV2D. The influence of eIF2A alteration on the initiation of individual PTEN isoforms.

These results collectively demonstrate that the expression of CUG^{816} initiated PTENε as well as the other two known isoforms PTENα and PTENβ is modulated by eIF2A.

Q3.16. *minor spelling correction. Please, correct FALG-eIF5 by FLAG-eIF5 on the first western blot.*

R3.16. We feel so obliged for the reviewer's careful review of our data, and we have changed the FALG-eIF5 in Fig 4C to FLAG-eIF5 in the revised version.

Q3.17. *Authors concluded from figure 4a-c: "Together, these data indicate that PTENε synthesis is closely regulated by the eIF2A-dependent mechanism." Please, avoid such overstatements that go beyond results since these data only prove that there is a correlation between PTENε and eIF2A levels. More experiments are needed in order to conclude that eIF2A regulates PTENε (i.e. genetic rescue using reintroduction of FLAG-eIF2A).*

R3.17. We feel very apologetic for the inaccuracy in describing the relationship between eIF2A and PTENε. As was shown in R3.15, the following experiments were carried out to further explore the correlation between PTENε and eIF2A.

(a) Accordingly, genetic rescue by reintroduction of FLAG-eIF2A was performed. As PTENε expression is downregulated markedly in eIF2A knockdown cells, in the revised version, we further measured the level of PTEN isoforms in eIF2A knockdown Hela cells transfected with FALG-eIF2A and it turns out that eIF2A

overexpression compensates for the downregulation of PTENε and other N-terminal extended PTEN isoforms resulting from knockdown of endogenous eIF2A.

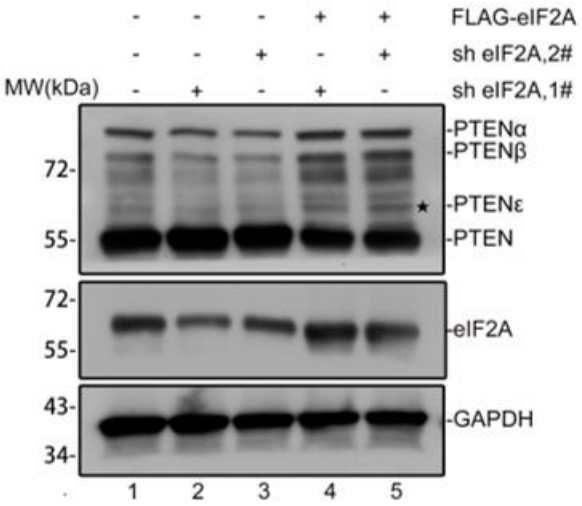


Fig EV2B. The reduced expression of PTEN isoforms in eIF2A knockdown cells was rescued by eIF2A reintroduction.

(b) We linked the 5'UTR of PTEN with luciferase reporter to testify the influence of eIF2A alteration on the translation of distinct isoforms initiated by specific upstream codons. Meanwhile, the non-AUG start codons that initiate other potential PTEN variants within the 5'UTR were mutated to CTC codons to ensure that only one isoform was expressed in each panel. Luciferase activity was measured with the Dual-Luciferase Assay System (Promega) following the manufacturer's protocol. We found that overexpression of eIF2A indeed promotes the translation of PTENα, PTENβ, and PTENε individually (Fig EV2D).

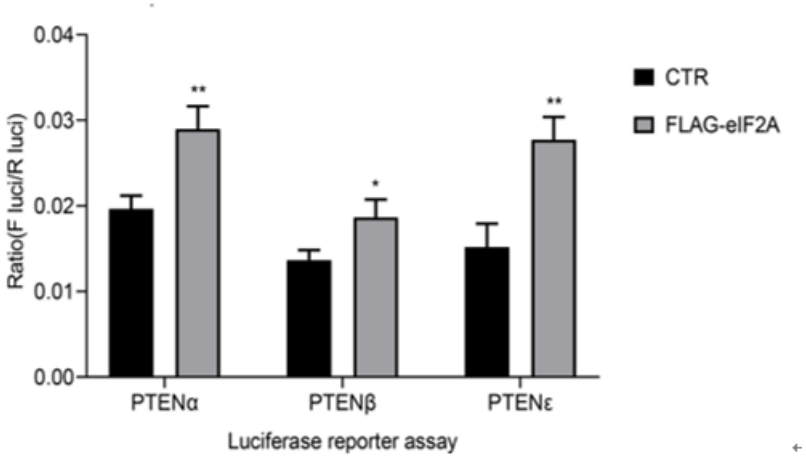


Fig EV2D. The influence of eIF2A alteration on the initiation of individual PTEN isoforms.

These results collectively demonstrate that the expression of CUG^{816} initiated PTENε as well as the other two known isoforms PTENα and PTENβ is modulated by eIF2A.

Q3.18. *Figure 6b: Do these proteins (FSCN1, ACTR2, CDC42 and VASP) interact with*

PTENα and PTENβ? In addition, it would be interesting, although not essential, to perform this IP experiment in PTEN KO HEK 293 cells to discard the fact that the complex that might be formed between the different overexpressed PTEN isoforms and the endogenous ones is responsible for the interaction with FSCN1, ACTR2, CDC42 and VASP.

R3.18. As was shown in Fig EV3A, we overexpressed C-terminal HA-tagged PTEN variants (PTENα, PTENβ, PTENε, and canonical PTEN) in HEK293 cells separately and determined whether these variants can interact with the filopodia formation-related proteins through co-IP. We found that only C-terminal HA-tagged PTENε could physically associate with endogenous VASP, FSCN1, CDC42, and ACTR2 while canonical PTEN and other two PTEN isoforms (PTENα and PTENβ) couldn't, suggesting that unlike other PTEN variants, PTENε can specifically associate with these filopodia-formation associated proteins.

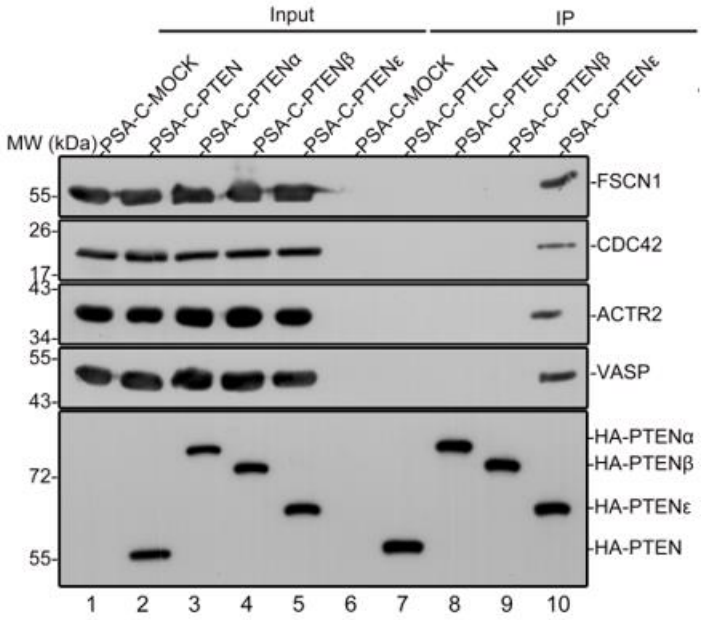


Fig EV3A. PTENε, but not other known PTEN isoforms, interacts with filopodia formation related proteins.

Considering that exogenously expressed PTENα, PTENβ, and canonical PTEN cannot physically interact with FSCN1, ACTR2, CDC42, and VASP, thus the assumption that overexpressed PTEN isoforms may form a complex with the endogenous ones that promotes the specific interaction between PTENε and these four filopodia formation related proteins may not conflict with our statement that PTENε is responsible for the interaction with FSCN1, ACTR2, CDC42, and VASP.

Q3.19. *Figure 6c: this experiment cannot be considered endogenous immunoprecipitation of PTENε since the different PTEN isoforms have been overexpressed. Please, modify this in the text and figure legend. Also, authors concluded from figure 6a-c: "which indicates that these proteins were PTENε targets"*

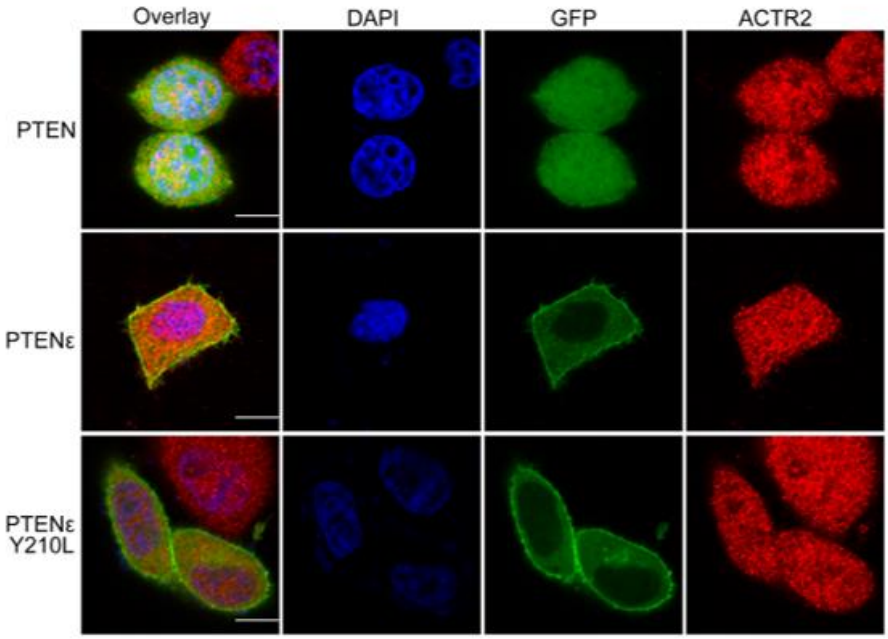
Please, avoid such overstatements that go beyond results since these data only prove that FSCN1, ACTR2, CDC42 and VASP interact with PTENε. As a suggestion, the authors could consider performing endogenous IP with anti-PTENα.

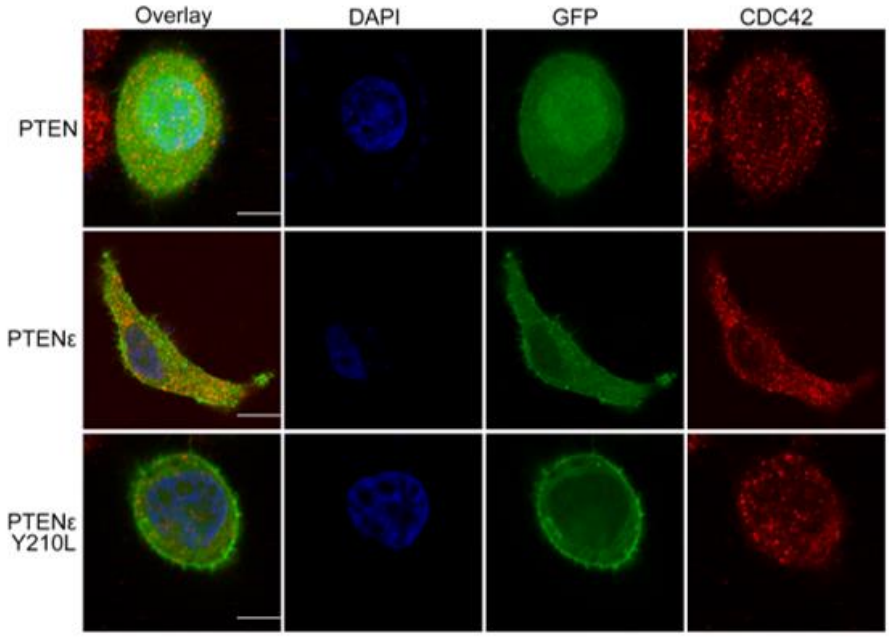
R3.19. We are grateful to the reviewer for such a careful review of our work, and we agree that this experiment cannot be considered endogenous immunoprecipitation of PTENε, we have remodified the relative content in the revised version and thoroughly inspected the entire manuscript to avoid similar overstatements. As was shown on page12, "…these interactions were further confirmed by endogenous immunoprecipitation…" have been changed to "…these interactions were further confirmed through immunoprecipitation with endogenous PTENε target proteins…", and so do the "… which indicates that these proteins were PTENε targets…".

Given that the anti-PTENα antibody was raised by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENα, performing endogenous IP with anti-PTENα is infeasible as all the N-terminal extended PTEN isoforms can be efficiently recognized by this anti-PTENα antibody.

Q3.20. *Figure 6d: authors claimed "immunofluorescence showed extensive co-localization of PTENε with these interactors in the membrane, whereas PTEN did not (Fig. 6D and Supplementary Fig. 6)". However, authors do not show the immunofluorescence micrographs showing canonical PTEN staining together with the interactors. Furthermore, do the endogenous CDC42, FSCN1, VASP, ACTR2 also colocalize with PTENε? And also, do CDC42, FSCN1, VASP, ACTR2 colocalize with PTENε Y210L?*

R3.20. We thank the reviewer for the insightful comments and detailed instructions. As suggested, plasmids expressing C-terminal GFP tagged PTEN, C-terminal GFP tagged PTENε and C-terminal GFP tagged PTENε Y210L were constructed and transfected in Hela *PTEN*$^{-/-}$ cells. Monoclonal antibodies (Santa Cruz, sc-8401(CDC42), sc-21743(FSCN1), sc-166103(ACTR2), and CST, 3132(VASP)) were used to label endogenous CDC42, FSCN1, ACTR2, and VASP separately. Membrane colocalization between endogenous CDC42, FSCN1, VASP, ACTR2, and PTENε was shown in Fig EV3B, and as to PTEN Y210L mutant and canonical PTEN group, there was no obvious or very little colocalization in the cell membrane between them and endogenous CDC42, FSCN1, VASP, or ACTR2 (Appendix Fig S4b), further supporting our statement that PTENε but not canonical PTEN or protein phosphatase deficient PTENε Y210L mutant physically associated with these filopodia formation proteins in the cell membrane.


Overlay
DAPI
GFP
ACTR2
PTEN
PTENε
PTENε
Y210L


Overlay
DAPI
GFP
CDC42
PTEN
PTENε
PTENε
Y210L

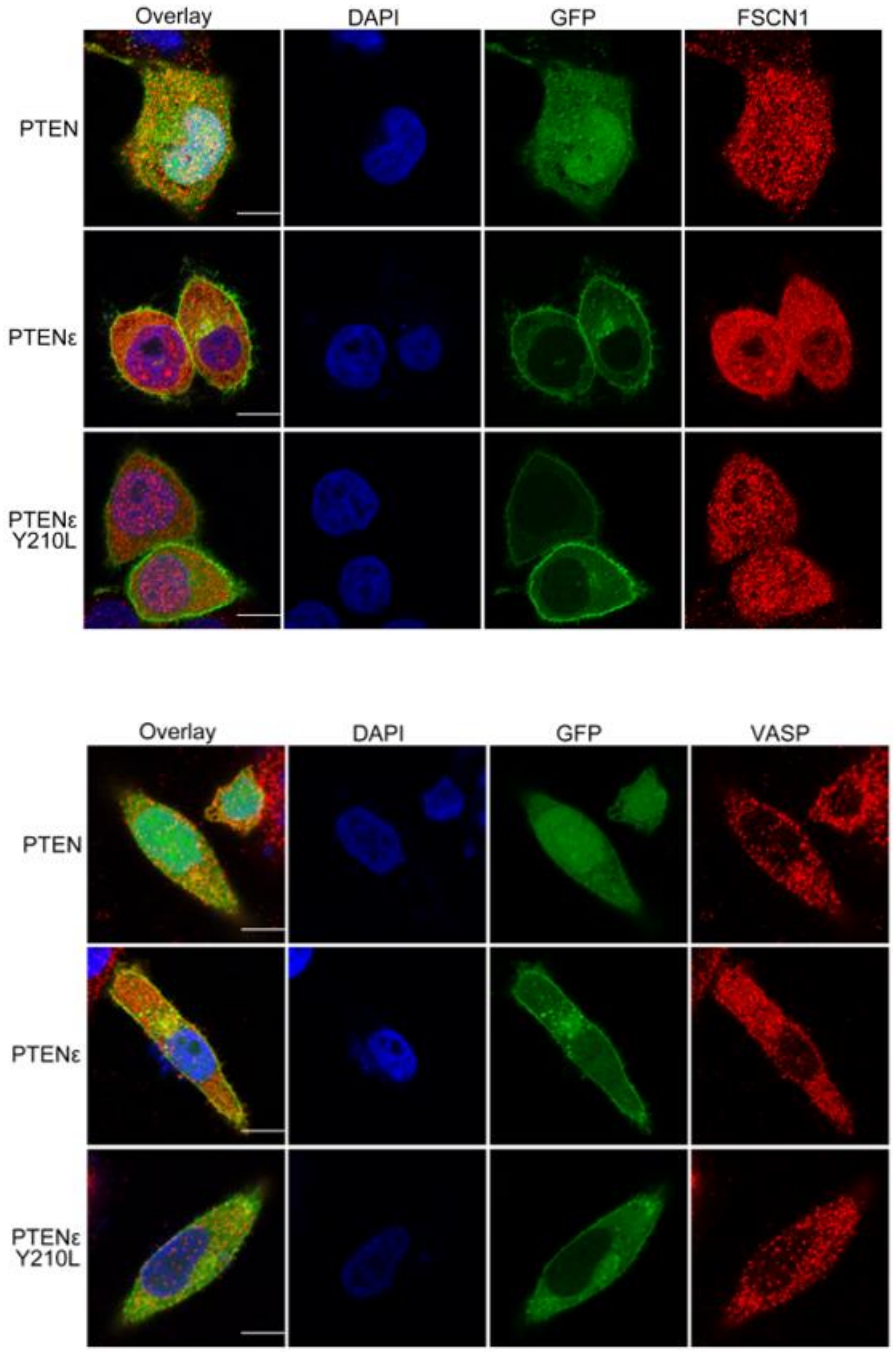


Fig EV3B and Appendix Fig S4b. Membrane colocalization of PTENε and endogenous partners in Hela cells.

Q3.21. *Figure 6f: Also, FSCN1 and CDC42 are reduced. Should be mentioned in the text (end of page 12). Also, the authors ignore these proteins for phosphorylation assays.*

R3.21. We thank the reviewer for the careful review of our work. The sentence "...while the interaction between PTENε and FSCN1 and CDC42 was also reduced after PTENε protein phosphatase abolishment, but to a much lower extent..." has been added in the revised version. Previously we performed in vitro kinase and phosphatase assay with

FSCN1 and CDC42 proteins. FLAG-FSCN1 and FLAG-CDC42 were purified with ANTI-FLAG® M2 Affinity Gel (F2426) and 3×FLAG Peptide (F4799) from HEK293 cells after transfection with pCMV-FSCN1 and pCMV-CDC42 for 30h. Then the corresponding kinase PKCα (FSCN1) or SRC (CDC42) and PTENε were added step by step according to methods shown in the manuscript. In the end, all these elutes were analyzed by Mass Spectrum to find out putative dephosphorylation sites mediated by PTENε. However, even though we performed these experiments many times by perfecting every step, we did not discover any consistent known sites involved in filopodia formation that can be dephosphorylated by PTENε. Maybe the specific effect of PTENε on FSCN1 and CDC42 related pathways and their role in filopodia formation remain further investigation.

Q3.22. *Figure 7f: Authors claimed "As shown in Fig. 7F, the number of migrated and invaded cells in Hela PTENε$^{+/-}$ was substantially reduced compared to WT cells". However, figure 7f shows that the number of migrated and invaded cells in Hela PTENε$^{+/-}$ was substantially increased compared to WT cells. This should be corrected.*

R3.22. We feel so obliged for your tremendous patience in reviewing our manuscript and we have checked the whole manuscript to make our statement accurate and clarified. As was shown in the revised manuscript on page 17, we have rewritten our statement.

Q3.23. *Supplementary Figure 1: It is advised to show the numbers of the nucleotides (position) and not just show the sequence.*

R3.23. We thank the reviewer for this valuable suggestion and we have added the corresponding numbers of the nucleotides (position) in the revised Appendix Fig S1.

Q3.24. *Supplementary Figure 4a: Not matched, does have GFP*

R3.24. Our apology for the misleading in Appendix Fig S3b (revised), as we have demonstrated in the manuscript and Appendix Fig S3b (revised), to eliminate the expression of GFP, we introduced a stop codon TAG just before the initiation site of GFP. So, although the GFP still exists on the plasmid as shown in the revised Appendix Fig S3b scheme, the stop codon TAG lied in front of this GFP would terminate its expression.

Q3.25. *Supplementary Figure 4a, lower panel in text: Suppl Figure 4b in figure*

R3.25. Our apology for our inattention and we are such grateful for your tremendous patience and preciseness, we have meticulously gone over the entire body of this article to avoid the same mistakes and changed the "…Supplementary Fig. 4a, lower panel…" to the proper content that is consistent with Appendix materials in the revised manuscript.

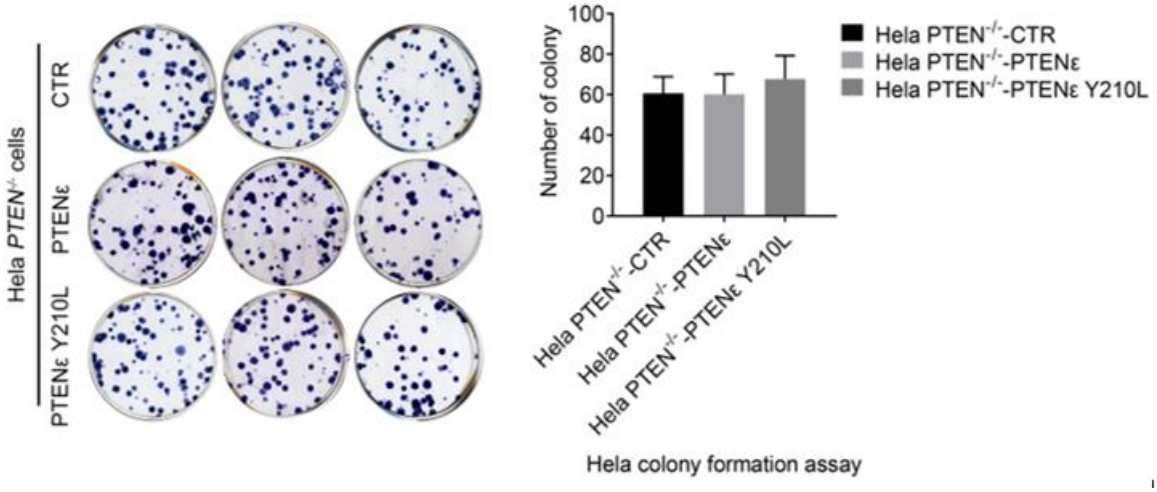
Q3.26. *Supplementary figure 8 a and b: is it consistent that PTENε Y210L is more expressed than PTENε both in H4 and in Hela cells? Also, in supplementary figure 8b,*

PTENε G210L should be changed to PTENε Y210L. What does PCDH stand for?

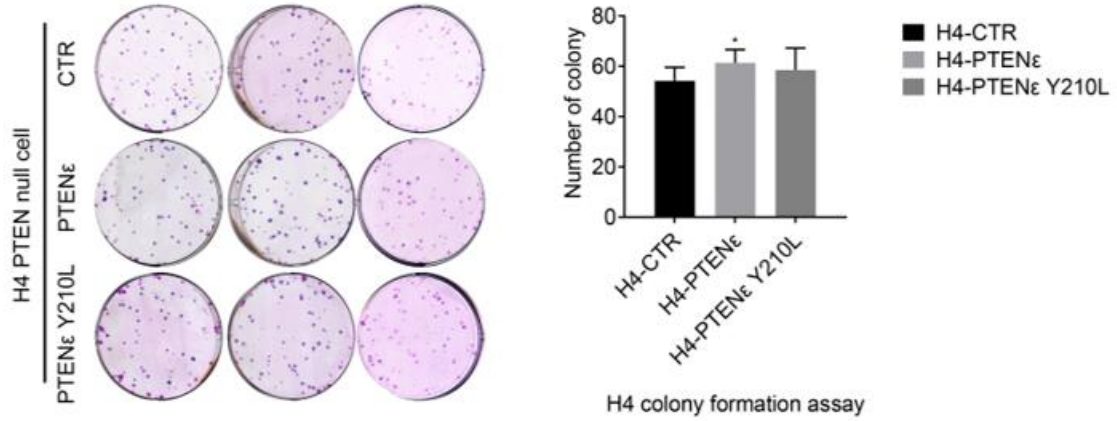
R3.26. We have carefully examined the whole paper to correct any potential mistakes and misleading. The misspelling PTENε G210L in primary Supplementary Fig 8b has been changed to PTENε Y210L and all the corresponding labels in the manuscript Figures have been scrupulously inspected. PCDH stands for the lentiviral backbone that we utilized to construct the stable transfected Hela *PTEN*$^{-/-}$ cells and the PTEN null H4 cells. The expression of PTENε Y210L is indeed higher than wild-type PTENε both in Hela and H4 stably transfected cells, considering that although there is much less PTENε expressed, the migrated and invaded cells in wild type PTENε panel were still far less than that in control or protein phosphatase dead PTENε mutant group, we thought this may not conflict with our statement that PTENε is involved in tumor metastasis suppression by its protein phosphatase activity.

Q3.27. *Supplementary figure 8c and d: it would be interesting to check colony formation in Hela and H4 cells when PTENε Y210L is incorporated. Also, there is a star with uncertain meaning that should be explained.*

R3.27. We apologize for the inaccuracy in the corresponding manuscript, in the revised version, "...Concerning cell growth, overexpression of PTENε did not affect, compared with control cells..." has been changed to "...Concerning cell growth, overexpression of wild-type PTENε or PTENε protein phosphatase deficient mutant hardly affects cell proliferation compared with the control group while PTENε slightly promotes the proliferation of H4 cells...". Hela *PTEN*$^{-/-}$ and H4 PTEN null cells stably transfected with wildtype PTENε, PTENε Y210L, or mock plasmid were used to check the influence of PTENε and its protein phosphatase on cell proliferation by colony formation assay. As was shown in the revised Appendix Fig S6c and d, neither wild-type PTENε nor PTENε phosphatase deficient mutant impacts the colony formation compared with the mock group in Hela *PTEN*$^{-/-}$ cells. But in H4 PTEN null cells, exogenously overexpressed PTENε slightly promotes the proliferation of H4 cells compared with the mock group. But there is no proliferation difference observed between PTENε and PTENε Y210L or PTENε Y210L and the mock group. This disparity may be due to cell type difference between Hela and H4 cells and given that the number of migrated and invaded cells in the PTENε panel is greatly less than that in the other two groups, we thought this may not conflict with the role PTENε played in suppressing tumor metastasis.



Appendix Fig S6c. Colony formation assay carried in PTENε and PTENε Y210L overexpressed Hela cells.



Appendix Fig S6d. Colony formation assay carried in PTENε and PTENε Y210L overexpressed H4 cells.

Q3.28. *End of page 12. The authors refer to "substracts": should be substrates*

R3.28. Accordingly, "substracts" has been changed to “substrates” in the revised version.

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1st Revision - Editorial Decision 23rd Dec 2020

Dear Dr Yin,

Thank you for submitting your revised manuscript (EMBOJ-2020-105806R) to The EMBO Journal. My apologies for getting back to you with delay due to protracted reviewer input. Your amended study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Regarding the remaining points of referee #3, we ask you to adjust data illustration and representation accordingly or introduce caveats where appropriate.

In addition, we need you to take care of a number of points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have further questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Introduce ORCID IDs for all corresponding authors (Y.Y.) via our online manuscript system. Please see below for additional information.

>> Please specify distinct author contributions for Y.J. .

>> Remove privacy from the datasets and the password from the 'Data availability" section.

>>Provide a synopsis image at higher resolution.

>> Change the title of the Appendix file ToC to "Appendix Table of Contents" and add the reference section. Remove Appendix Fig S9-S12 from the appendix file and upload as source data zipped and provided as one file per figure. Adjust the 'data availability' section and author checklist accordingly.

>> Avoid textual redundance with your 2017 study.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Mar 2021.

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Referee #1:

The authors have satisfactorily addressed all of my points. This is an interesting study and is ready to be published in EMBO journal.

Referee #2:

authors have addressed all my concerns, and should be accepted for publication

Referee #3:

Overall, this is a comprehensive study which provides sufficient evidence for the author´s conclusion and should be published.

Although the authors addressed most of the concerns and comments of this reviewer in a thorough manner, the revised version of the manuscript requires some clarification with regards to data analysis and interpretation.
1. A schematic representation of all the isoforms described (such as the one included by the authors on the rebuttal letter R3.5) should be incorporated in the manuscript. Some of the new isoforms are marginally mentioned, but represent an important finding. Also, these new and non characterized isoforms which appear with question marks in R3.5 on the rebuttal letter should be indicated as PTEN3, PTEN4 and PTEN6, since authors retain the nomenclature of PTEN5 for their isoform of interest.
2. It would be highly recommended that authors show an inset panel on figure EV4F at a higher magnification to really appreciate differences in cell morphology.
3.In figure R3.3 (linked to figure 2A): It is unclear how the relative ratio of endogenous PTEN isoforms versus the overexpressed was calculated, given that the western blot bands of exogenous PTEN are not individually visible (it shows as a smear). However, it would be important to include the western blot data in the manuscript for a qualitative comparison of the exogenous levels of PTEN versus the endogenous ones.
4. In reply to Question 3.20, authors included a worrisome figure, that was not included as such in figures EV3B and Appendix Fig S4b, regarding the membrane colocalization of PTENε and endogenous partners in Hela cells. First, the colocalization of PTENε and endogenous partners at the cell membrane is not that obvious and not so different from the colocalization with PTEN Y210L. However, colocalization is more evident at other cellular compartments, such as the cytoplasm. Therefore, the images included do not support the conclusions drawn by the authors. Second, a very worrying alteration of the signal levels was detected in the green and red channels (clearly visible when splitting channels of the merged image using dedicated software) in different panels of the figure attached to R3.20. In addition, this figure has not been incorporated in the paper as it is shown in the rebuttal letter, which makes it difficult to compare the colocalization between PTEN and PTENε with the endogenous candidates, as it is split between main and supplementary figures. Also, the stainings of PTEN Y210L were completely omitted in the manuscript. Figure R3.20 should be revised, presented with equivalent exposure and unaltered intensity, and the conclusions drawn should be adjusted. With the present daya this reviewer does not observed a discreet co-localization of the target proteins with PTENe, compared to PTENwt and Y210L. All three PTEN variants should be included in a single data panel.
5. In appendix figure S6d, it is unclear how with the small mean difference, the relatively high data dispersion (SEM is presented) and the low number of biological replicates (n=3) the difference between control and PTENe is $p<0.05$. More details of the statistics and a dot plot instead of an

histogram should be presented.

Point-by-point response

We would like to thank all three reviewers for their supportive comments and insightful suggestions. We have now addressed the comments by reviewer #3 as follows.

Reviewer #1

The authors have satisfactorily addressed all of my points. This is an interesting study and is ready to be published in EMBO journal.

Reviewer #2

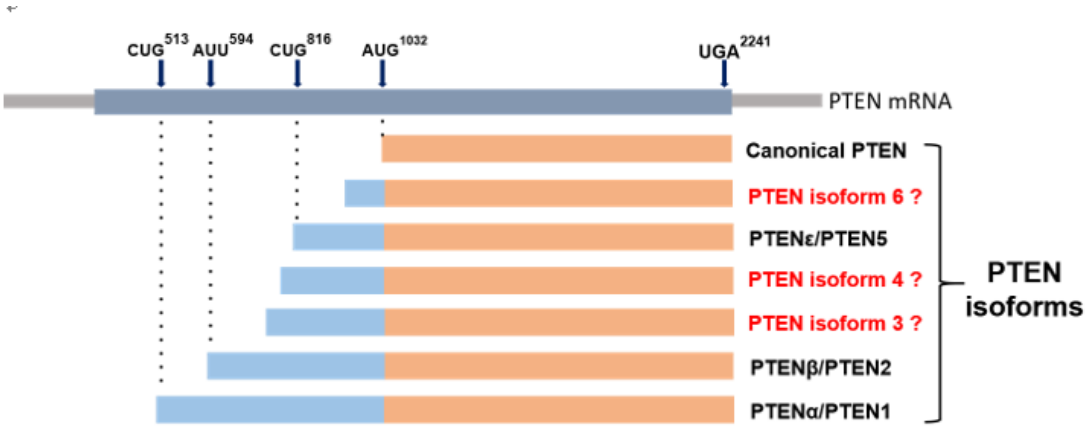
Authors have addressed all my concerns, and should be accepted for publication.

Reviewer #3

Overall, this is a comprehensive study which provides sufficient evidence for the author´s conclusion and should be published.

Q3.1. *A schematic representation of all the isoforms described (such as the one included by the authors on the rebuttal letter R3.5) should be incorporated in the manuscript. Some of the new isoforms are marginally mentioned, but represent an important finding. Also, these new and non-characterized isoforms which appear with question marks in R3.5 on the rebuttal letter should be indicated as PTEN3, PTEN4 and PTEN6, since authors retain the nomenclature of PTEN5 for their isoform of interest.*

R3.1. Thank you for your insightful comment and instruction. We have incorporated an explicit schematic explanation of isoforms translated from the PTEN gene in the revised Appendix Fig S9. The non-characterized PTEN isoforms are indicated as PTEN3, PTEN4, and PTEN6 in the diagram as suggested.



Appendix Fig S9. A schematic representation of the PTEN family members. The proteins labeled with the question mark "?" stand for unidentified isoforms of PTEN that remain to be further verified.

Q3.2. *It would be highly recommended that authors show an inset panel on figure EV4F at a higher magnification to really appreciate differences in cell morphology.*

R3.2. As suggested, we have added an inset panel at a higher magnification in the revised Fig EV4F to better illustrate the influence of endogenous PTENε depletion on cell morphology.

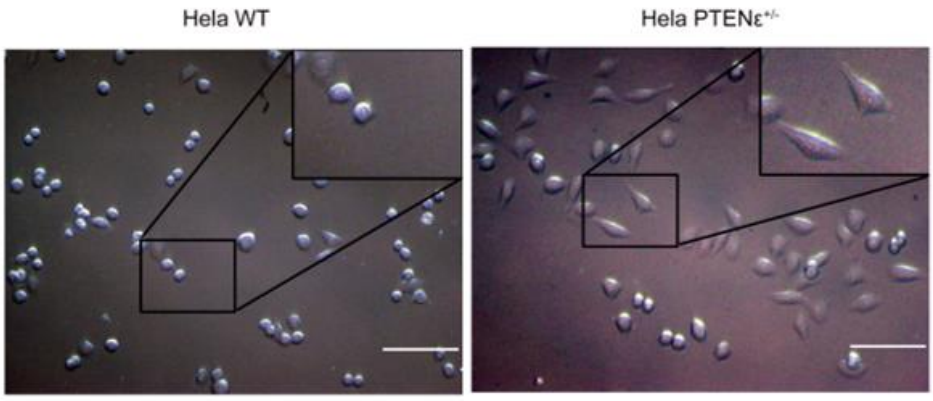
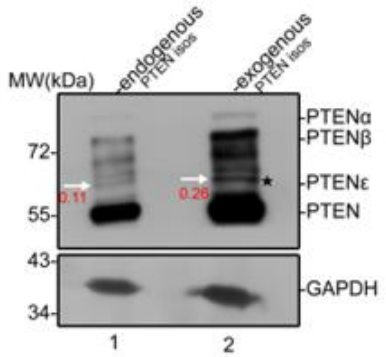


Fig EV4F. The influence of endogenous PTENε depletion on cell morphology.

Q3.3. *In figure R3.3 (linked to figure 2A): It is unclear how the relative ratio of endogenous PTEN isoforms versus the overexpressed was calculated, given that the western blot bands of exogenous PTEN are not individually visible (it shows as a smear). However, it would be important to include the western blot data in the manuscript for a qualitative comparison of the exogenous levels of PTEN versus the endogenous ones.*

R3.3. Thank you for the careful review of our data and we appreciate the advice to improve our manuscript. To compare relative levels of exogenous PTEN isoforms versus the endogenous ones, equal amounts of cell lysates were loaded onto 6% SDS-PAGE gels. When 50ug of cell lysates were loaded, the protein bands in immunoblots of exogenous PTEN isoforms were distinct, whereas the endogenous PTEN isoforms could hardly be detected as their expression is relatively low. When 150ug of cell lysates were loaded, the protein bands in immunoblots of endogenous PTEN isoforms were clearly appeared, whereas the exogenous PTEN isoforms were not individually visible. Finally, as shown in Fig R3.3, we observed that the bands of both exogenous PTEN isoforms and the endogenous ones were relatively distinct when 100ug of cell lysates were loaded, although the bands of the exogenous group were slightly unsharp. The immunoblots of Fig R3.3 in the rebuttal letter have been

added in the revised Appendix Fig S2. Moreover, the protein bands used to quantitate the relative level of endogenous PTENε versus the overexpressed one were indicated with arrows as suggested.

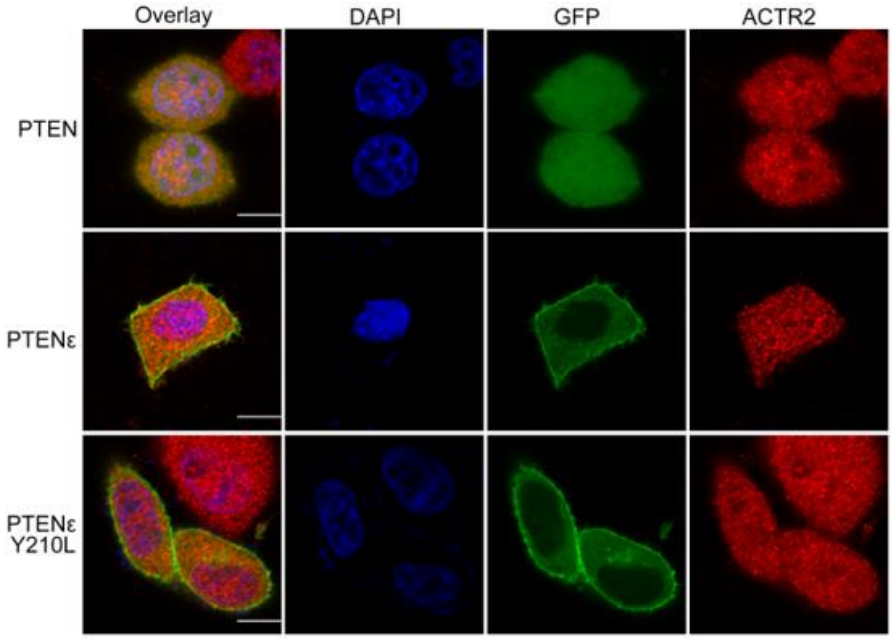


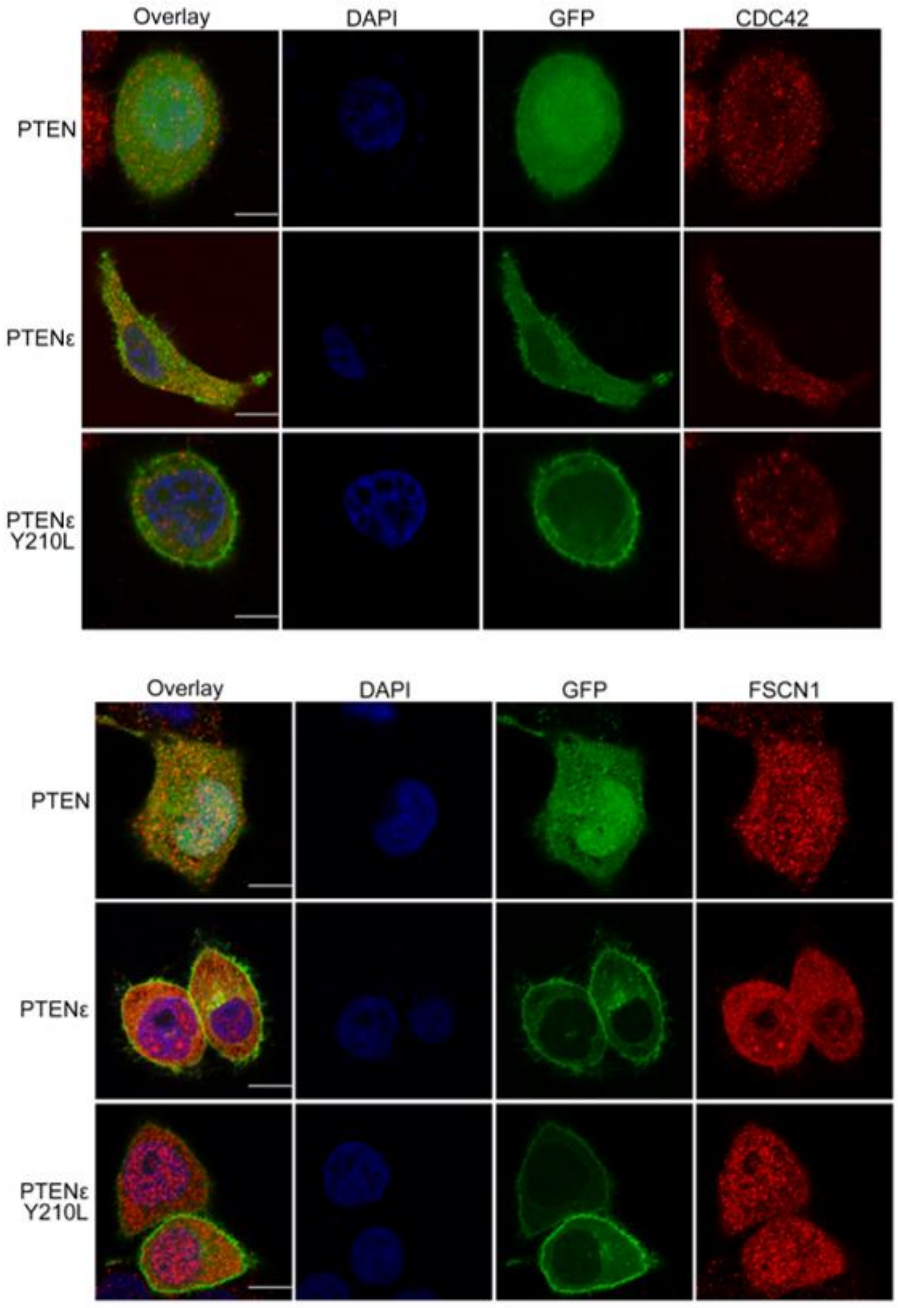
Appendix Fig S2 (Fig R3.3). The expression of endogenous PTENε and the overexpressed one.

Q3.4. *In reply to Question 3.20, authors included a worrisome figure, that was not included as such in figures EV3B and Appendix Fig S4b, regarding the membrane colocalization of PTENε and endogenous partners in Hela cells. First, the colocalization of PTENε and endogenous partners at the cell membrane is not that obvious and not so different from the colocalization with PTEN Y210L. However, colocalization is more evident at other cellular compartments, such as the cytoplasm. Therefore, the images included do not support the conclusions drawn by the authors. Second, a very worrying alteration of the signal levels was detected in the green and red channels (clearly visible when splitting channels of the merged image using dedicated software) in different panels of the figure attached to R3.20. In addition, this figure has not been incorporated in the paper as it is shown in the rebuttal letter, which makes it difficult to compare the colocalization between PTEN and PTENε with the endogenous candidates, as it is split between main and supplementary figures. Also, the stainings of PTEN Y210L were completely omitted in the manuscript. Figure R3.20 should be revised, presented with equivalent exposure and unaltered intensity, and the conclusions drawn should be adjusted. With the present data this reviewer does not observed a discreet co-localization of the target proteins with PTENe, compared to PTEN wt and Y210L. All three PTEN variants should be included in a single data panel.*

R3.4. Thank you for the careful examination of our data and we agree that the conclusions drawn should be adjusted. As shown below, the pictures in Figure R3.20 were revised and presented with equivalent exposure and unaltered intensity as suggested. Partial colocalization between endogenous filopodia formation-related proteins and C-terminal GFP tagged PTENε in the membrane can be observed, while C-terminal GFP tagged PTENε Y210L and C-terminal GFP tagged PTEN also show colocalization with these proteins, especially in the cytoplasm. Thus, we agree with the reviewer that the corresponding statement in the manuscript is inaccurate, and in

the revised version “immunofluorescence reveals the extensive co-localization of PTENε with these partners in the membrane, whereas canonical PTEN did not, further confirming the specificity of the interaction of PTENε with these membrane-localized proteins.” (Page 12, line 17 continuing to line 19) has been replaced with “Also, immunofluorescence reveals that PTENε partially colocalizes with these partners in the cell membrane, further confirming the interaction of PTENε with these membrane-localized proteins.”. Accordingly, the images of PTEN and PTENε Y210L were not included in figures EV3B in order to be in line with the statement in the revised manuscript. This change does not affect the conclusions that PTENε interacts with these filopodia formation-related proteins.




Overlay
DAPI
GFP
CDC42
PTEN
PTENε
PTENε Y210L
Overlay
DAPI
GFP
FSCN1
PTEN
PTENε
PTENε Y210L

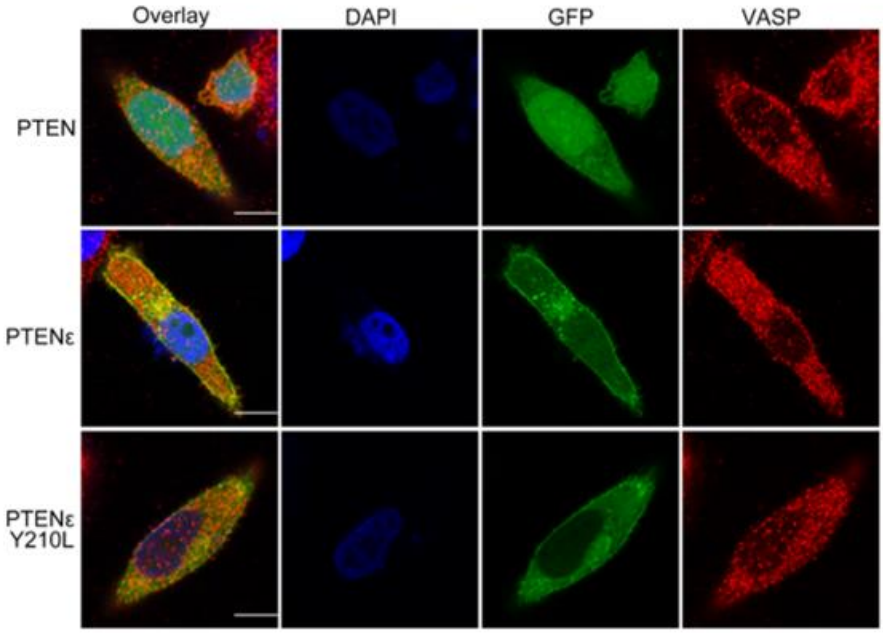
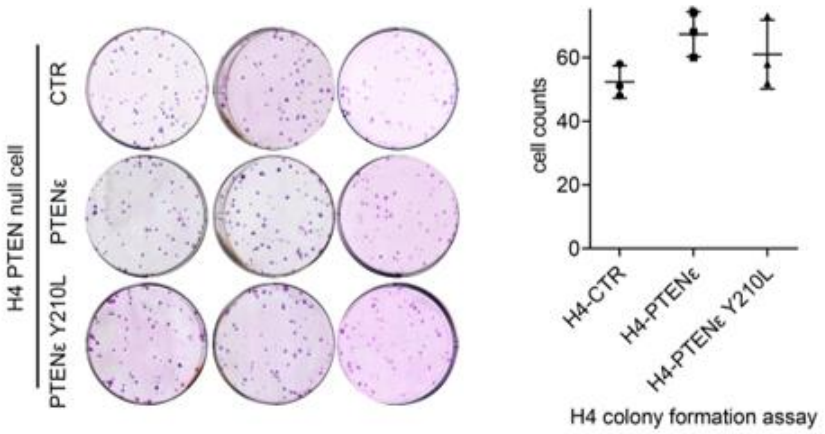


Fig EV3B and Fig R3.1. Membrane colocalization of PTENε and endogenous partners in Hela cells.

Q3.5. *In appendix figure S6d, it is unclear how with the small mean difference, the relatively high data dispersion (SEM is presented) and the low number of biological replicates (n=3) the difference between control and PTENe is p<0.05. More details of the statistics and a dot plot instead of an histogram should be presented.*

R3.5. When evaluating the influence of PTENε overexpression on cell proliferation in H4 cells, cells of each group (CTR, PTENε or PTENε Y210L) were seeded onto three wells of a 6 well plate, and three independent experiments were performed. Image J software was used to automatedly calculate the colony number in each well, and the mean value of cell colonies of each group in every independent experiment was used as input and analyzed by the two-tail unpaired t-test (Prism GraphPad software v8.0). The details of the statistics were added in the corresponding figure legends, and the histogram was replaced with a dot plot as suggested. Data were presented as the mean ± SD instead of the mean ± SEM in the revised manuscript.



Appendix Fig S7d. Colony formation assay carried out in PTENε and PTENε Y210L overexpressed H4 cells.

2nd Revision - Editorial Decision 1st Feb 2021

Dear Dr Yin,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional rebuttal figures included in this file.

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Kind regards,

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Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105806R

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

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes were determined according to the previously published studies by us or other groups and power analyses were not performed in our manuscript.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample sizes in our manuscript are similar or identical to previously published studies in the relative field.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analyses.
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.	Cultured cells were randomly allocated to different experimental groups and the animals used in our studies were allocated to different groups by blinding of the investigators(two people carried out this experiment, one people randomly allocated the animals while the other performed the experiment without knowing exactly the experimental groups.)
For animal studies, include a statement about randomization even if no randomization was used.	The animals used in our studies were allocated to different groups by blinding of the investigators(two people carried out this experiment, one people randomly allocated the animals while the other performed the experiment without knowing exactly the experimental groups.)
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, the animals we used to evaluate the function of PTENε on tumor metastasis in vivo were mixed together when we bought them from Beijing Vital River Laboratory Animal Technology Co. and then all the mice were randomly divided into three groups by one author. Finally, another author performed this experiment without knowing the exact group name of each group.
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?	Yes, when we choose the statistical methods to analyze the corresponding results, we consulted relative papers, and the test used for each data set is specified in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA

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Is the variance similar between the groups that are being statistically compared?	If the variance was not similar, we performed statistical analyses that were not affected by dissimilar variance.

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).	Yes, the detailed information required for the antibodies was supplemented in the Appendix table S1.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes, the cell lines used in our studies were authenticated by STR locus analysis and were 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.	Yes, as was shown in the Materials and Methods section, the C57BL/6 (6weeks, male) were purchased from Vital River Laboratory Animal Technology. All animals were maintained in a special pathogen-free facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, as was shown in the Materials and Methods section, All animals mentioned above were maintained in a special pathogen-free facility, and the animal study protocols used were approved by the ethics committee of Peking University Health Science Center (approval number LA2017142)
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.	Mouse experiments follow the "Guide for the Care and Use of Laboratory Animals"(National Academy Press, USA, 1996).

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
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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	Yes, as was shown in the manuscript Data availability section, nucleotide sequence data reported in this work are available in the Genebank databases under the accession numbers MK783286 (Homo sapiens) and MK783287 (Mus musculus). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository with the data set identifier PXD022245 (http://www.ebi.ac.uk/pride/archive/projects/PXD022245). The original immunoblots, polyacrylamide gels, and agarose gels are provided in the source data file. Primers used in this study are listed in Appendix Table S2, S3, and S4. All other data supporting the findings of this study are available within the article or its supplementary information or from the corresponding author upon reasonable request.
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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
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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA