EMBO reports

Hexokinase 2 displacement from mitochondria-associated membranes prompts Ca^{2+}-dependent death of cancer cells

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

1st Editorial Decision 25 September 2019

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but reading the reports it becomes clear that a significant revision will be required to substantiate the findings. HK2 localization to MAMs needs to be further analysed and verified. Peptide treatment seems to cause a redistribution of HK2 within membranes rather than its relocalization into the cytosol and this should be further investigated. Also the effect of the peptide on mitochondrial metabolism should be analysed. A better documentation of the in vivo experiments is necessary and the role and localization of HK2 in other cancer cell lines and non-transformed cells should be tested.

In addition, referee 1 and referee 3 noted that no mechanistic insight is provided. How does HK2 displacement influence intracellular Ca2+ fluxes and what are the physiological cues that would induce its displacement from MAMs? I agree that further insight along these lines would strengthen the manuscript but if the observations are substantiated as outlined above, a full mechanistic understanding is not necessary for publication in EMBO reports. Please note that we also publish Articles without a restriction on the number of figures or the length of the text.

Given the overall constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

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

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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

can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

10) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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

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REFEREE REPORTS

Referee #1:

This manuscript is a complement of a previous study of the same lab describing the apoptotic effects of HK2 when dissociated of mitochondria. In the present work, the authors show that mitochondrial dissociation of HK2 results in the release of Ca++ from the ER, entry of Ca++ to the mitochondria, and calpain-mediated apoptosis. Finally, they show that the inhibition of the association of HK2 to the MAMs by using a peptide-based strategy, inhibits the growth of cancer cells in vitro and in vivo. The experiments are well designed and the data is conclusive. The originality of the study, however, is precluded by the previous publication of the lab (https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0001852&type=printable). This work would have more relevance if the mechanisms underlying the effects of HK2 in mitochondria were addressed. In addition, I have the following critics and concerns.

1. The peptide does not change the enzymatic activity of HK2. But is this enzymatic activity, in addition to the association with MAMs, required to induce apoptosis in cancer cells? The use of HK2 mutants without hekokinase activity but that still retain the ability to associate in MAMs could further help to elucidate how HK2 regulates ER and mitochondrial function.

2. It is well described by these authors and others that HK2 is associated to mitochondria and that this is important for cell survival. Is it known which are the specific physiological or pathological conditions or stresses that causes the dissociation of HK 2? If not, this could also contribute to elucidating the function of HK2 in mitochondria.

3. The analysis of the glucose metabolism, for instance Seahorse analyses or equivalent, in the cells

expressing the inhibitory peptide are important to better describe the specificity of the treatment.

4. To validate the specificity of the peptide treatment in the cells, the same experiment could be performed I HK2-/- cells.

5. In the figure 5, the authors show that there is no tumor regression in response to the treatment, although the tumor growth is significantly decreased. The question is: if the peptide induces cancer cell death, a decrease in tumor volume would be expected. Maybe some cancer cells are resistant to the treatment or alternatively, the peptide did not reach these cancer cells. Can the authors analyze the presence of the peptide in the treated tumors? Apoptosis could also be tested in these tumors.

6. All over the manuscript, the experiments are not very well described. For instance, the figure 3 is very confusing. What each panel represents is not clear. It is the same data shown in different ways (graphic bars, curves, immunofluorescent images, FACS...). Not even in the figure's legends is described. Moreover, important information is sometimes missing. For instance, in figure 5 there is no indication of how long the treatment was. In 5G-H, the X-axis is days, hours? How many mice were used in the study? In M&M the authors say that male and female mice were used. The results shown are male or female?

Referee #2:

The authors have analyzed the precise localization of hexokinase 2 (HK2) in cells and they have characterized the effects of a HK2-targeting peptide to validate it as a potential anti-neoplastic strategy. The methods used are appropriate, and data are interesting. Some of the results obtained require additional experimental support.
Major comments.
1. The SPLICS methodology of analysis of ER-mitochondria contacts was initially set up using a short-range (SPLICSS) and a longer-range probe (SPLICSL) (Cieri et al., 2018). However, authors have only used SPLICSL to proof that HK2 codistribute with MAMs. Based on the heterogeneity of MAMs, it would be relevant to document whether HK2 localized in tighter MAMs.
2. Incubation with cl-HK2pep caused a rapid displacement of HK2 in puncta that do not codistribute with SPLICSL. The authors indicate that HK2 translocates to cytosol. However, this was not tested and again HK2 localized in puncta. Another alternative is that HK2 moves to a mitochondrial region free from ER. This should be analyzed by using biochemical techniques or microscopy assays.
3. The observation that the HK2-targeted peptide causes calcium entry through plasma membrane suggests the existence of unespecificity or alternatively that the effects of the peptide at the plasma membrane are secondary of the ER effects. The authors should analyze this to potentially discard nonspecific effects of the peptide.
4. Does the cl-HK2pep cause alterations in cellular ATP or in the rate of anaerobic glycolysis?
5. Five intratumor injections of HK2 peptides reduced the size of allograph-injected colon cancer cells or breast cancer cells. It is key that the authors provide information on the physiological status of mice receiving the cl-HK2pep or HK2pep. Was there any difference between mice receiving the more specific versus the generic peptide?

Referee #3:

The manuscript suggests that HK2 is associated with MAMs in tumor cells and that this localization is relevant for tumor cell metabolism. At MAMs, HK2 would influence intracellular Ca2+ fluxes, thus controlling cellular metabolism and death. A large portion of the manuscript deals with a synthetic peptide that the authors claim displaces HK2 specifically in tumor cells and then causes them to undergo rapid cell death.
The topic of the manuscript is interesting and the paper is well written and easy to follow, but at the moment, there are serious shortcomings in the manuscript and the design of the experiments. Important information is missing at the moment and listed in my specific points. While I would like to sound more positive, the manuscript appears rushed and/or is maybe better suited for a journal with a longer manuscript format, not necessarily of lower impact, where the increased space may lead to a more positive outcome. It is possible that the authors have some of the data needed to make this story more convincing, but could not add it due to space limitations.

Specific points

1. The characterization of tumor tissue is not providing much information. Figure 1A-C is of unclear importance to the story, since we do not know how this positive staining is reflected in healthy tissue. Is it more or less? And if so, what would it mean?
2. Similarly, the localization of HK2 to MAMs is certainly relevant and very interesting to this reviewer. However, in the current manuscript as per the title and abstract of the paper, the authors suggest this is somehow decisive in the tumor format, but only analyze it in HeLa cells, a cervical cancer cell line. This is not sufficient.
3. Most importantly, it is not clear WHY HK2 would influence intracellular Ca2+ fluxes. Does it associate with mitochondrial or ER Ca2+ handling proteins? Which ones? Under which conditions? What effects are observed? At the moment, this observation raises much more questions than it answers.
4. Do altered glucose levels in the culture medium influence any of the observations?
5. As per the recent standard methods and characterization paper by Scorrano et al., multiple approaches, also of a biochemical nature, should accompany MAM targeting assays. The paper currently contains none.
6. The localization of HK2 after peptide treatment is unclear. Together with point 4, this is even more of a concern, since under this condition, puncta appear, which are typically indicative of membrane association of SOME kind. It appears that the cleaved peptide causes a relocation with the mitochondrion or the ER, but where to?
7. The mechanism of HK2 relocation to the cytosol (or wherever it relocates to) has not been examined. Also, is its relocation observed in non-transformed cells?
8. Xestospongin-C also inhibits SERCA. This is a big concern here, since the authors pretreat their cells. Decreased loading of the ER under this condition could account for all observed effects.
9. The role of HK2 and the inhibitory peptide in non-transformed cells has not been investigated.
10. The cell death observed with the HK2 peptide is extremely rapid, suggestive of a mechanism neither involving HK2, nor caspases, speaking as the "devil's advocate". At a minimum, the authors should investigate the role of necrosis and altered HK2 expression in their models. Also, what happens in non-transformed cells? How would titrations in both models look like?

1st Revision - authors' response 23 December 2019

Point-by-point reply to the Referees' comments (in red and Italics)

Referee #1:

This manuscript is a complement of a previous study of the same lab describing the apoptotic effects of HK2 when dissociated of mitochondria. In the present work, the authors show that mitochondrial dissociation of HK2 results in the release of Ca++ from the ER, entry of Ca++ to the mitochondria, and calpain-mediated apoptosis. Finally, they show that the inhibition of the association of HK2 to the MAMs by using a peptide-based strategy, inhibits the growth of cancer cells in vitro and in vivo. The experiments are well designed and the data is conclusive. The originality of the study, however, is precluded by the previous publication of the lab (https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0001852&type=printable). This work would have more relevance if the mechanisms underlying the effects of HK2 in mitochondria were addressed. In addition, I have the following critics and concerns.
We thank the reviewer for these comments, which gave us the possibility to improve the quality of the manuscript. As a general statement, the present manuscript precisely identifies the cellular sub-compartment hosting HK2, i.e. MAMs, and unveils the molecular consequences of its removal from MAMs, thus mechanistically dissecting the effect of the HK2-targeting peptide. In our opinion this set of data, together with the design and testing of an activatable peptide that opens therapeutic perspectives, confers a high degree of originality to the present manuscript, and renders it a development more than a "complement" of our previous work. This said, we agree with the importance of all concerns raised by the reviewer, which we address in the following point-by-point reply.

1. The peptide does not change the enzymatic activity of HK2. But is this enzymatic activity, in addition to the association with MAMs, required to induce apoptosis in cancer cells? The use of HK2 mutants without hexokinase activity but that still retain the ability to associate in MAMs could further help to elucidate how HK2 regulates ER and mitochondrial function.
The use of HK2 mutants raised a conceptual point: if such mutants are expressed in a background where expression of wild-type HK2 is not completely ablated, even a very low number of such proteins could interact with the peptide, setting off an amplifying response barely distinguishable from the one observed in the absence of mutants (see also the reply to point 4). Therefore, we used an alternative strategy to evaluate the role of HK2 enzymatic activity in apoptosis induction of cancer cells: we used the peptide on cancer cells either placed in a medium with no glucose or, more stringently, in the presence of two different HK2 inhibitors, 5-thio-glucose and 2-deoxyglucose. In all these conditions, cell death induction by the peptide was indistinguishable from that observed in cells harbouring the active enzyme. These data have been added in the revised paper as Figure EV4A-B.

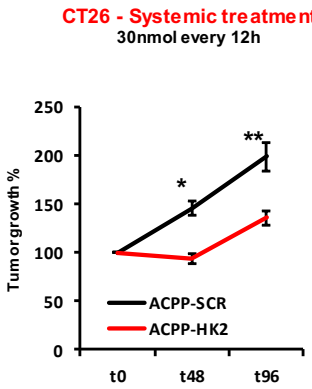
2. It is well described by these authors and others that HK2 is associated to mitochondria and that this is important for cell survival. Is it known which are the specific physiological or pathological conditions or stresses that causes the dissociation of HK 2? If not, this could also contribute to elucidating the function of HK2 in mitochondria.
A variety of conditions favour HK2 dissociation from mitochondria (reviewed in Roberts and Miyamoto, Cell Death Differ, 2015, 22 248). Death stimuli, such as Bax/Bak pro-apoptotic proteins, competitively HK2 binding to mitochondria. PHLPP (PH domain leucine-rich repeat protein phosphatase) dephosphorylates and inhibits Akt and antagonizes Akt-dependent phosphorylation of Thr473 on HK2, required for its interaction with mitochondria. Indeed, this phosphorylation decreases the sensitivity of HK-II to G-6P induced mitochondrial dissociation, probably the main allosteric regulation of HK2 mitochondrial binding. This regulation is made more complex by Akt-dependent inactivation of GSK3beta, the activity of which inhibits HK2 binding to the outer mitochondrial protein VDAC. Similarly, we have found that a molecular complex including DMPK and Src favors HK2 interaction with mitochondria in muscle-derived cells, and inhibiting these interactions prompts HK2 dissociation (Pantic et al, Cell Death Dis 2013; 4: e858). This fine tuning could be relevant not only in cancer, but also in other pathological conditions, such as diabetes (e.g. Gurel et al, Mol Biol Rep. 2013;40:4153). This has been briefly added in the text (Introduction section).

3. The analysis of the glucose metabolism, for instance Seahorse analyses or equivalent, in the cells expressing the inhibitory peptide are important to better describe the specificity of the treatment.
Our peptide does not affect the enzymatic activity of hexokinases (both isoform 1 and 2), either on purified enzymes or in cells, as demonstrated in Fig. 1I-J and EV2C (numbering of the revised manuscript). So, it can be better defined as a displacing peptide, more than an “inhibitory peptide”. Nonetheless, we agree that it is interesting to assess whether it affects glucose metabolism. However, it must be considered that the peptide kills the majority of target cells in few minutes (see for instance Fig. 3 and Fig. 4), and to study its possible metabolic effects we need to use it at much lower concentrations. We have performed extracellular acidification rate experiments after giving cells 200 nM peptide, the highest possible concentration that does not prompt cell death in the first hour of treatment, without observing any effect on these bioenergetic parameters. These data are now included as Figure EV2D. See also the response to point 4 of Reviewer 2.

4. To validate the specificity of the peptide treatment in the cells, the same experiment could be performed I HK2-/- cells.
We have tried to address this important point by knocking-out HK2 expression through CRISPR/Cas9 technology in our tumor cell models. Unfortunately, we were unable to obtain any knock-out cells: they rapidly die in a spontaneous way, possibly because of the importance of HK2 for their viability. Moreover, the peptide was effective, albeit less, on tumor cells where HK2 expression was only knocked-down. We believe that this is due to the amplifying effect of detaching even few HK2 molecules form MAMs, eliciting a massive Ca^{2+} flux into mitochondria. We have therefore used an alternative strategy to assess the specificity of the peptide effect, using on non-transformed hepatocytes, which do not express HK2. We have found that the peptide is completely ineffective on hepatocytes, and these data are now included as Figure EV5Q.

5. In the figure 5, the authors show that there is no tumor regression in response to the treatment, although the tumor growth is significantly decreased. The question is: if the peptide induces cancer cell death, a decrease in tumor volume would be expected. Maybe some cancer cells are resistant to

the treatment or alternatively, the peptide did not reach these cancer cells. Can the authors analyze the presence of the peptide in the treated tumors?
Apoptosis could also be tested in these tumors.
The effect of the peptide in vivo on solid tumors is a complex issue, which involves taking into account several variables that could explain why we do not observe a decrease in tumor volume but only a decrease in tumor growth (which we believe is an important result in any case). A possible list of such variables is the following:
a) the quantity of the injected peptide could be insufficient to obtain the maximal effect on the tumor mass. Accordingly, a quantity of injected peptide lower than that shown in Figure 5 (30 nmol/injection versus 60 nmol/injection) has a lower effect, but statistically significant, on the tumor (compare Fig. 5 with the attached figure).
However, it was not possible to inject higher peptide quantities in mice because of solubility limits. We can probably overcome such limits by changes in peptide amino acid sequence;



b) the peptide was injected in pulses, every 24 hours. For safety and welfare of animals it was not possible to increase the frequency of such pulses. As the peptide rapidly induces cell death, it is possible that after a surge of effect (apoptosis), tumor cells that are not targeted have the time to start over their growth and proliferation;
c) the rate of proliferation of these xenografted tumor cells largely exceeds that of the majority of human tumor types. In a therapeutic perspective, this leads to an underestimation of the possible real effect of the peptide,
d) it was very difficult to study both the pharmacodynamics of the peptide, as we should assess HK2 detachment from MAMs or some downstream effects (raises in intracellular Ca^{2+}, mitochondrial depolarization) that unfold very rapidly, and apoptosis induction, as cells undergo a peculiar, caspase-independent apoptosis program.
We believe that a thorough investigation of all these aspects deserves a further study aimed at identifying the optimal conditions to begin a preclinical pipeline for bringing the peptide closer to a therapeutic tool. However, we have included a morphological evaluation of apoptosis and mitosis performed on ex vivo tumor samples, showing a statistical significant effect of the peptide (Supplementary Figure EV5D of the revised manuscript). The absence of a massive apoptosis induction is in accord with the lack of a major decrease in tumor volume, and can be explained both by the previously mentioned points and by the fact that a morphological inspection is a snapshot that does not catch all processes, underestimating for instance rapid events of apoptosis that occurred hours before sample retrieval.
Finally, we would like to highlight that our peptide has a dramatic and extremely promising effect on non-solid tumor cells, namely primary B-CLL cells freshly obtained from patients (Figure 4).

6. All over the manuscript, the experiments are not very well described.
For instance, the figure 3 is very confusing. What each panel represents is not clear. It is the same data shown in different ways (graphic bars, curves, immunofluorescent images, FACS...). Not even in the figure's legends is described.
Moreover, important information is sometimes missing. For instance, in figure 5 there is no indication of how long the treatment was. In 5G-H, the X-axis is days, hours?
How many mice were used in the study? In M&M the authors say that male and female mice were used. The results shown are male or female?
We agree with the reviewer's comment. We have now extended the legend to Figure 3, added the timing of Fig. 5 experiments and included the required information about mice.

Referee #2:

The authors have analyzed the precise localization of hexokinase 2 (HK2) in cells and they have characterized the effects of a HK2-targeting peptide to validate it as a potential anti-neoplastic strategy. The methods used are appropriate, and data are interesting. Some of the results obtained require additional experimental support.
We thank the reviewer for these comments, to which we have responded by the following point-by-point reply.

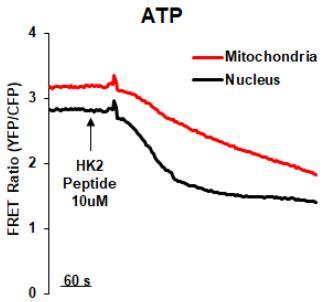
Major comments.

1. The SPLICS methodology of analysis of ER-mitochondria contacts was initially set up using a short-range (SPLICSS) and a longer-range probe (SPLICSL) (Cieri et al., 2018). However, authors have only used SPLICSL to proof that HK2 codistribute with MAMs. Based on the heterogeneity of MAMs, it would be relevant to document whether HK2 localized in tighter MAMs.
This observation gave us the opportunity to better analyze HK2 localization in MAMs. We have now repeated experiments with the short-range probe (SPLICSs), finding that HK2 does not localize in tighter MAMs. These data have been added as Figure EV1C.

2. Incubation with cl-HK2pep caused a rapid displacement of HK2 in puncta that do not codistribute with SPLICSL. The authors indicate that HK2 translocates to cytosol. However, this was not tested and again HK2 localized in puncta. Another alternative is that HK2 moves to a mitochondrial region free from ER. This should be analyzed by using biochemical techniques or microscopy assays.
We agree with the Reviewer that we cannot be sure that the peptide causes HK2 redistribution from MAMs into cytosol. This assumption was based on previous data, obtained by subcellular fractionation analyses (e.g. Chiara et al, PLos ONE, 3, e1852, 2008), and by the less punctuate distribution of HK2 following a short-term peptide treatment, which is particularly visible in Figure EV2B. However, it must be underlined that even a partial relocation of HK2 (as quantified by the Pearson's coefficient in the same Figure 1H and EV2B) is sufficient to trigger an amplifying series of events rapidly inducing apoptosis, making difficult to precisely assess where a small portion of HK2 translocates to; whereas in the longer period, the peptide elicits a major disruption of intracellular architecture, again hampering a refined dissection of HK2 localization. Therefore, in order to avoid unproven statements, we have eliminated any indication of HK2 translocation to cytosol.

3. The observation that the HK2-targeted peptide causes calcium entry through plasma membrane suggests the existence of unespecificity or alternatively that the effects of the peptide at the plasma membrane are secondary of the ER effects. The authors should analyze this to potentially discard nonspecific effects of the peptide.
We believe that peptide treatment induces Ca^{2+} entry through the plasma membrane as a secondary effect of ER Ca^{2+} release, as HK2pep rapidly prompts an increase in mitochondrial Ca^{2+} even in virtual absence of extracellular Ca^{2+}, i.e. in the presence of EGTA (Figure 2F). The same experiment indicates that Ca^{2+} entry through the plasma membrane is responsible for the subsequent increase of mitochondrial Ca^{2+} levels. This has been added in the text.

4. Does the cl-HK2pep cause alterations in cellular ATP or in the rate of anaerobic glycolysis?
As per response to the point 3 of reviewer 1, we have performed both oxygen consumption rate extracellular acidification rate experiments giving cells 200 nM peptide, the highest possible concentration that does not prompt cell death in the first hour of treatment, without observing any effect on these bioenergetic parameters. These data are now included as Figure EV2D. We have also used a FRET approach to assess ATP levels following peptide treatment. We observe a slow decline, which is apparently secondary to the fast induction of mitochondrial depolarization (see the Figure attached below).



5. Five intratumor injections of HK2 peptides reduced the size of allograph-injected colon cancer cells or breast cancer cells. It is key that the authors provide information on the physiological status of mice receiving the cl-HK2pep or HK2pep. Was there any difference between mice receiving the more specific versus the generic peptide?
We rigorously follow international guidelines to assess the clinical score of treated animals in order not to overcome humane endpoints, according to a protocol approved from Italian Health Ministry (authorization number: 547/2016-PR), as stated in the Methods section. We monitor any possible sign of pain, including ataxia, alopecia, ulceration of the neoplastic mass and weight loss, and we could detect none of them in our animal cohorts, either treated with intratumor or intraperitoneal injections of scrambled or HK2pep peptides (Fig. 5F and 5G-H, respectively). We have included these considerations in the text and the animal weight analysis as Figure EV5B. In Figure EV5C the lack of any evident damage to a variety of organs is also reported.

Referee #3:

The manuscript suggests that HK2 is associated with MAMs in tumor cells and that this localization is relevant for tumor cell metabolism.
At MAMs, HK2 would influence intracellular Ca2+ fluxes, thus controlling cellular metabolism and death.
A large portion of the manuscript deals with a synthetic peptide that the authors claim displaces HK2 specifically in tumor cells and then causes them to undergo rapid cell death.
The topic of the manuscript is interesting and the paper is well written and easy to follow, but at the moment, there are serious shortcomings in the manuscript and the design of the experiments.
Important information is missing at the moment and listed in my specific points. While I would like to sound more positive, the manuscript appears rushed and/or is maybe better suited for a journal with a longer manuscript format, not necessarily of lower impact, where the increased space may lead to a more positive outcome. It is possible that the authors have some of the data needed to make this story more convincing, but could not add it due to space limitations.
We thank the Reviewer for these comments, and we appreciate that he/she finds the topic interesting and the manuscript well written. Nonetheless, for the sake of clarity we need to answer to a general comment made by the Reviewer. We never affirm in the manuscript that HK2 localization in MAMs "is relevant for tumor cell metabolism" or "controls cellular metabolism". This would be an overstatement of the general consensus about the fact that HK2 induction, and not its subcellular localization, in a variety of tumor cell types plays an important role in their metabolic rewiring (see for instance Mathupala and Pedersen, 2010, Biochim Biophys Acta 1797: 1225-1230). In the Discussion, we only suggest that HK2 localization in MAMs can play a role in controlling intracellular Ca^{2+} fluxes. However, this is not the core result of our work, but a speculation resulting from a body of data provided in the manuscript that show how displacing HK2 from MAMs elicits a fast and massive change in Ca^{2+} levels in ER, cytosol and mitochondria (this point is investigated with a variety of technical approaches all along the manuscript, and we do not think it is a "claim"). In other words, studying whether HK2 at MAMs affects intracellular Ca^{2+} fluxes is not the topic of the manuscript, but a potentially interesting perspective that stems from this work, which is instead focused on defining the precise localization of HK2 in tumor cells, on dissecting the downstream effects of its displacement from this localization and on opening a consequent therapeutic perspective. Probably, a misunderstanding on this point led the Reviewer to state that the manuscript appears "rushed".
This said, we agree on the importance of several specific comments made by the Reviewer, to which we provide in the following a point-by-point reply.

Specific points

1. The characterization of tumor tissue is not providing much information. Figure 1A-C is of unclear importance to the story, since we do not know how this positive staining is reflected in healthy tissue. Is it more or less? And if so, what would it mean?
We agree with the reviewer that IHC inspections of tumor samples do not provide much information on the relative level of HK2 in tumor vs healthy tissue. Such a quantification is quite challenging, as it would be necessary to study HK2 levels in a healthy tissue that are not always easy to obtain. Ideally, it would be necessary to analyze HK2 levels in neoplastic and non-neoplastic regions of the same sample. Similar data exist in literature (e.g. Katagiri et al, Histol Histopathol (2017) 32:351; Sato-Tadano et al, Cancer Sci 2013; 104:1380; more in general, see https://www.proteinatlas.org/ENSG00000159399-HK2). For this reason, and as the IHC image was only intended to show that the peptide target is present in a variety of tumor types, IHCs were removed, with the exception of HK2 staining in B-CLL. In fact, the effect of the peptide was further investigated in primary B-CLL samples, where it has shown extremely promising results (Fig. 4). Therefore, we have compared HK2 expression in reactive lymphoid tissues and B-CLL samples, and added this IHC investigation as Figure 4A of the revised manuscript. Western blot analyses and IF inspections of HK2 expression/localization are shown as positive controls for all further experiments and were therefore maintained in the manuscript (WB were moved to Figure EV1A of the revised manuscript).

2. Similarly, the localization of HK2 to MAMs is certainly relevant and very interesting to this reviewer. However, in the current manuscript as per the title and abstract of the paper, the authors suggest this is somehow decisive in the tumor format, but only analyze it in HeLa cells, a cervical cancer cell line. This is not sufficient.

We have shown HK2 localization to MAMs of HeLa cells (Fig. 1B-C, Fig. 1H and Fig. EV1D; numbering of the revised manuscript), colorectal carcinoma COLO741 cells, plexiform neurofibroma PN 04.4 cells, breast adenocarcinoma MDA-MB 231 cells and malignant peripheral nerve sheath tumor S462 cells (Fig. EV1B, numbering of the revised manuscript). Quantification of these experiments is reported in Fig. 1D-F.

3. Most importantly, it is not clear WHY HK2 would influence intracellular Ca2+ fluxes. Does it associate with mitochondrial or ER Ca2+ handling proteins? Which ones? Under which conditions? What effects are observed? At the moment, this observation raises much more questions than it answers.
The role played by HK2 at the interface between ER and mitochondria is an important issue raised by our work. However, the focus of the manuscript is on the effect of HK2 detachment form MAMs (see also our general comment to this Reviewer), and we think that understanding whether and how HK2 tunes Ca^{2+} fluxes between ER and mitochondria in pathophysiological conditions is an ambitious objective that largely exceeds the scope of this work. Nonetheless, we have found a co-immunoprecipitation between HK2, of which about 80% localizes in MAMs in the different tumor cell models (Fig. 1F), and GRP75, a chaperone that directly interacts with IP3R at MAMs and favors mitochondrial Ca^{2+} uptake upon IP3-dependent ER Ca2+release (Filadi et al, Cell Calcium, 62, 1, 2017). This experiment has been added as Figure EV3I and paves the way for further investigations.

4. Do altered glucose levels in the culture medium influence any of the observations?
Cells placed in a medium with no glucose did not undergo cell death for at least 2 hours, and cell death induction by the peptide was indistinguishable from that observed in cells placed in a medium with glucose. These data were added as Figure EV4A (see also the response to point 1 of Reviewer 1).

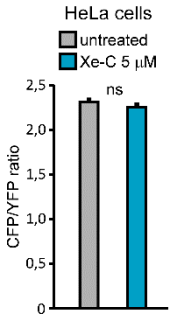
5. As per the recent standard methods and characterization paper by Scorrano et al., multiple approaches, also of a biochemical nature, should accompany MAM targeting assays. The paper currently contains none.
We have added immunoprecipitation analyses showing a direct interaction between HK2 and the MAM protein GRP75 (Figure EV3I of the revised manuscript; see also response to point 3).

6. The localization of HK2 after peptide treatment is unclear. Together with point 4, this is even more of a concern, since under this condition, puncta appear, which are typically indicative of membrane

association of SOME kind. It appears that the cleaved peptide causes a relocation with the mitochondrion or the ER, but where to?
This is an important concern, also raised by Reviewer 2; please see our reply to his/her point 2.

7. The mechanism of HK2 relocation to the cytosol (or wherever it relocates to) has not been examined. Also, is its relocation observed in non-transformed cells?
The active portion of HK2pep is composed by the first 15 amino acids of the HK2 sequence, which are believed to be responsible of its interaction with mitochondria; however, the interaction partner(s) of HK2 on mitochondria remain poorly characterized. Unveiling the protein(s) which HK2 bind to at MAMs, and if these interactions are dynamically modulated in different biological conditions of the cell, or in certain pathophysiological settings, is an exciting and major project (see also reply to point 3, and our added experiment showing HK2/GRP75 interaction) that we would like to tackle. However, we think that this can be the objective of future studies. Importantly, we have found (Figure EV4N of the revised version) that the fraction of HK2 localized in MAMs is extremely low in non-transformed cells, where HK2pep is poorly effective and does not induce any HK2 relocation.

8. Xestospongin-C also inhibits SERCA. This is a big concern here, since the authors pretreat their cells. Decreased loading of the ER under this condition could account for all observed effects.
We have used the same FRET approach of Figure 2A to evaluate whether Xestospongin-C inhibits SERCA, finding that it does not display any effect per se on ER Ca^{2+} levels. This experiment is attached below.



9. The role of HK2 and the inhibitory peptide in non-transformed cells has not been investigated.
Technically speaking, our peptide is not an inhibitory one, as it does not influence HK2 activity (see Figure 1 and our response to point 3 of Reviewer 1). Nonetheless, by dose-response and kinetic experiments we have shown that it has a very limited activity as an apoptosis inducer on non-transformed cells, such as mouse macrophage RAW 264.7 cells and mouse myoblast C2C12 cells (Fig EV4O-P of the revised manuscript) and hepatocytes (Fig EV4Q of the revised manuscript).

10. The cell death observed with the HK2 peptide is extremely rapid, suggestive of a mechanism neither involving HK2, nor caspases, speaking as the "devil's advocate". At a minimum, the authors should investigate the role of necrosis and altered HK2 expression in their models. Also, what happens in non-transformed cells? How would titrations in both models look like?
Experiments reported in Figure 3, Figure 4 and Figure EV4 demonstrate that the peptide rapidly induces a form of caspase-independent, calpain-dependent apoptosis, as cells undergo a process involving a sequence of apoptotic traits (mitochondrial depolarization, phosphatidylserine exposure on the cell surface and loss of plasma membrane integrity – for the sequence of events see Fig. 3D) that is not affected by a pan-caspase inhibitor, while it is blunted by a pan-calpain inhibitor. Loss of plasma membrane integrity without PS exposure on the cell surface, a typical hallmark of necrosis, was never observed (see cytofluorimetric experiments in Fig 3 and Fig 4). For non-transformed cells, please see our rebuttal to point 9. All cells were treated with at least two peptide concentrations or more (see for instance Fig. 4F) for different time points.

2nd Editorial Decision 14 February 2020

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript but we have only recently received the last referee report and I have discussed these further with the referees.

As you will see, referee 1 and 2 acknowledge that the revised manuscript has addressed most of their concerns but referee 1 and 3 also point out the the mechanism by which HK2 modulates Ca2+ flux remains unexplained. In addition, referee 3 raises other important concerns, regarding the specificity of Xestospongin-C, the significance of the peptide inhibitor on tumor growth and insufficient evidence that HK2 localizes to MERCs.

Upon further discussion of these points with all the referees, we agreed to give you the opportunity to address the concerns regarding the pleiotropic drug Xestospongin-C that should be replaced or complemented with a different approach. Moreover, the conclusion that HK2 localizes to MAMs should be complemented with an additional, quantitative approach. All control experiments need to be provided. In addition, please address these and the other concerns in a complete point-by-point response and by appropriate textual changes/discussion in the manuscript. The elucidation of the mechanism is not necessary at this point. Acceptance of the manuscript will depend on a positive outcome of a second round of review.

When you submit a revised manuscript, please also address these editorial issues:

- Your article will be published as Report. Therefore, please combine the Results and Discussion section.

- Please submit your manuscript as a .docx file.

- Please add a callout to Movies EV5 - EV9 in the text.

- Movies: please remove their legends from the manuscript file and provide them as README.txt file. The legend and its movie are then zipped together and uploaded as zip file. The correct nomenclature is Movie EVx.

- Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- You specify the Ethics Committee that approved the experiments involving human patients in the Author Checklist but not in the manuscript. Please add this information [This study was authorized by the Padua Ethics Committee (Approve Code: n. 3529/AO/14)] to the methods section.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

****

REFEREE REPORTS

Referee #1:

In this revised version of the manuscript, the authors have addressed some of my critics and suggestions. The quality of the manuscript has improved. The mechanisms explaining how HKII regulates mitochondrial processes remain, however, unanswered.

Referee #2:

The authors have adequately answered my prior questions so now I consider it deserves publication.

Referee #3:

Ciscato et al. have revised their manuscript attempting to answer the points raised by three reviewers. While some improvements have been made, I am still detecting serious issues with the manuscript. The most critical are 1) a lack of clear information of HK2 association with mitochondria-ER contacts (MERCs), 2) no information about how HK2 controls Ca2+ flux, thus making the study descriptive, 3) remaining concerns about the use of the pleiotropic xestospongin C and 4) serious questions about the significance of the peptide inhibitor on tumor growth.

Specific points

1. There appears to be a variability of the staining pattern regarding the localization of HK2. For instance, the pattern is different between 1A and 1B.
2. In this context, the extent of MAM inclusion is still confusing. MAM is a biochemical isolate of MERCs. Without assaying its composition, any statement of MAM "localization" is incorrect. It also raises the question: Is HK2 showing distinct extents of localization between MAM and mitochondria, as suggested by the previously mentioned distinct staining pattern? Confocal microscopy cannot distinguish between these two possibilities, it lacks the necessary resolution. What does it mean it is found on loose MERCs, but not tight MERCs (via SPLICSL vs. SPLICSS)?
3. Moreover, the loss of the SPLICSL overlap versus the loss of mitochondrial overlap in Figs 1H and EV2B should be compared to get an idea which localization is predominantly targeted by the peptide.
4. The co-IP of HK2 with Grp75 is insightful, but currently not integrated into the remainder of the manuscript. Is the HK2-targeted peptide able to disrupt the interaction? Note also that the interaction with Grp75 does not provide direct evidence of MAM association as this protein is mitochondrial. Also, what is the significance of this interaction? Is HK2 potentially blocking this Ca2+ signaling gateway? A test should be done with mutant HK2, as suggested by Reviewer #1.
5. It is unclear from the text what mechanism leads to the increase of cytosolic IP3 in Figure EV3A.
6. A test of xestospongin C under resting conditions is not insightful and does not mitigate concerns based on multiple publications with this reagent. These can be found in many papers outlining its shortcomings (especially the paper by de Smet and Parys 1999 and the recent study by Saleem and Taylor from 2014). The inhibition of SERCA concomitant with IP3R inhibition as shown in the response to my previous concern will not lead to a change in ER Ca2+. Instead, a better control would be within the setup of experiment 2A.
7. The claim that HK2 is induced as per Figure 4B is inadmissible without a control. The remainder of Figure 4 is compromised due to the lack of comparison between high and low expressing cells, as well as the lack of comparison between high and low HK2 association with MAMs.
8. The analysis of tumor growth for 3 days as done in Figure 5 is not insightful. Standard experimentation for solid tumors uses longer time frames than this. This unusual approaches, especially in the light of "normalization of growth to 100%" (how much did the volumes actually increase in this very short time frame???) raises serious questions.

Additional Communication 12 March 2020

Thank you for sending a point-by-point response to the remaining concerns from referee 3. I have meanwhile received feedback from referee 1 and 2 on it. Both referees considered your rebuttal convincing and concluded that the current evidence for HK2 localization is sufficient to merit publication in EMBO reports.
I should also note that we have meanwhile received a clarification from referee 3 regarding the potential unspecific effect of xestospongin C. The referee considers a text passage regarding potential issues with this drug sufficient to address his/her concerns.

It will therefore not be necessary to perform biochemical experiments to add further evidence for the localization of HK2 to MAMs. Please address all other concerns in the text/discussion and please provide tumor volumes for Figure 5.

2nd Revision - authors' response 22 March 2020

Referee #3.

Ciscato et al. have revised their manuscript attempting to answer the points raised by three reviewers. While some improvements have been made, I am still detecting serious issues with the manuscript. The most critical are 1) a lack of clear information of HK2 association with mitochondria-ER contacts (MERCs), 2) no information about how HK2 controls Ca2+ flux, thus making the study descriptive, 3) remaining concerns about the use of the pleiotropic xestospongin C and 4) serious questions about the significance of the peptide inhibitor on tumor growth.
Response. We thank the reviewer for considering our manuscript improved, and for giving us the opportunity to better explain these specific issues. In the following, we provide a detailed response to each of them: we address the association between HK2 and MAMs/MERCs in the response to reviewer's points 1-5; we discuss the use of Xestospongin C in the response to reviewer's point 6, and we answer to the concerns about the peptide effect on tumor growth addressing reviewer's points 7 and 8.
As a general point, we respectfully but resolutely disagree with the use of the term "descriptive" for the present manuscript. This work precisely identifies a death signalling pathway involving HK2 displacement from MAMs (an unprecedented subcellular localization for this enzyme), mitochondria Ca^{2+} overload elicited by IP3R opening in the ER, calpain activation and permeability transition pore opening in mitochondria.

Reviewer's point 1. There appears to be a variability of the staining pattern regarding the localization of HK2. For instance, the pattern is different between 1A and 1B.
Response. "Different" is a qualitative adjective, which makes difficult to answer to this point. In our opinion, the same punctate pattern over mitochondria can be observed for HK2 in both Fig. 1A and 1B, as well as in all our figures. It must be underlined that MAMs (aka MERCs) are dynamic structures, and it is impossible to find two single cells with an identical pattern of MAMs, or of MAM proteins. It is possible that the different magnification between Fig. 1A and 1B, as well as the different colors used to highlight HK2, made difficult a direct comparison between these two panels.

Reviewer's point 2. In this context, the extent of MAM inclusion is still confusing. MAM is a biochemical isolate of MERCs. Without assaying its composition, any statement of MAM "localization" is incorrect. It also raises the question: Is HK2 showing distinct extents of localization between MAM and mitochondria, as suggested by the previously mentioned distinct staining pattern? Confocal microscopy cannot distinguish between these two possibilities, it lacks the necessary resolution. What does it mean it is found on loose MERCs, but not tight MERCs (via SPLICSL vs. SPLICSS)?
Response. In order to assess HK2 localization at MAMs we have used three different approaches: classical confocal microscopy, by merging anti-HK2 antibody signals and mitochondria-directed probe staining; a split-GFP-based probe for ER mitochondria contacts termed SPLICS; co-immunoprecipitation between HK2 and the MAM protein GRP75. Even though we agree that each of these techniques could have some pitfalls, all experiments concur in finding HK2 in MAMs. Here we need to highlight the following important points:
a) SPLICS constitutes a state-of-the-art approach to study interactions between ER and mitochondria;
b) the percentage of HK2 dots in MAMs detected by the $SPLICS_L$ analysis is extremely high in all neoplastic cell models analyzed (about 80%, Fig. 1F), whereas it is much lower in non-neoplastic cells (about 35%, Fig. EV4N), strongly arguing against the measurement of a false positive signal in tumor cells;
c) as pointed out by the referee, HK2 better co-localizes with $SPLICS_L$ than with $SPLICS_S$. We think this is a further evidence of signal specificity, as $SPLICS_L$ staining identifies loose MERCs, where the distance between ER and OMM is < 40-50 nm, whereas $SPLICS_S$ stains tight MERCs, with a distance between ER and OMM < 8-10 nm. Considering the high probability of multimeric

interactions involving HK2 at MAMs, and the fact that the tetrameric structure of IP3Rs, which is one possible HK2 interacting partner, by itself protrudes outside ER membrane for ~13 nm (Prole and Taylor, Cold Spring Harb Perspect Biol 2019; 10.1101/cshperspect.a035063), we can envisage that HK2 is better accommodated in the looser MERCs.
d) it is not known any protein that exclusively localizes in MAMs and, as already mentioned, MAMs are extremely dynamic structures. Therefore, classical biochemical approaches (e.g. co-immunoprecipitations) are not exhaustive for assessing that any protein is in MAMs. Nonetheless, we found a co-immunoprecipitation between HK2 and Grp75 (Figure EV3I). Grp75 localizes both in MAMs, where it forms a complex with IP3Rs on the ER side and with VDAC1 on the mitochondrial side, and inside the mitochondrial matrix. As this latter district is separated by two membranes from MAMs, we believe reasonable that the observed interaction between Grp75 and HK2 occurs at the ER/mitochondria contact sites. Moreover, it is known that HK2 associates with VDAC1, suggesting that it may interact with the IP3Rs-grp75-VDAC1 complex. This could explain the preferential HK2 localization on loose MERCs, as these multimers could not settle in tight MERCs for obvious steric hindrance reasons. We have better explained these points in the text.

Reviewer's point 3. Moreover, the loss of the SPLICSL overlap versus the loss of mitochondrial overlap in Figs 1H and EV2B should be compared to get an idea which localization is predominantly targeted by the peptide.
Response. We had already addressed this concern in our original submission by measuring the Pearson's co-localization coefficient within the merge panels both in Fig. 1H and EV2B. A direct, quantitative comparison between these two measurements is poorly informative, as these are distinct experiments with different technical approaches; moreover, the Pearson coefficient is an internal correlation that can hardly be extrapolated and compared in different experiments.

Reviewer's point 4. The co-IP of HK2 with Grp75 is insightful, but currently not integrated into the remainder of the manuscript. Is the HK2-targeted peptide able to disrupt the interaction? Note also that the interaction with Grp75 does not provide direct evidence of MAM association as this protein is mitochondrial. Also, what is the significance of this interaction? Is HK2 potentially blocking this Ca2+ signaling gateway? A test should be done with mutant HK2, as suggested by Reviewer #1.
Response. We fully agree with the reviewer that finding an interaction between HK2 and Grp75 opens several questions about its biochemical meaning. However, we believe that a thorough dissection of HK2 interacting partners in MAMs, their dynamic modulation under different cellular conditions, the possible role played by HK2 in handling Ca^{2+} fluxes in this district, as well as the consequences of such modulation for the biological processes of neoplastic cells, are fascinating issues disclosed by the present manuscript that need to be tackled in a further and ambitious project. Here, we have added this experiment mainly to strengthen the information about MAM localization of HK2 (please refer to the previous response for this specific issue), but its potential importance goes beyond a simple association, as Grp75 is involved in mitochondrial Ca^{2+} uptake upon IP3-dependent ER Ca^{2+}release (Szabadkai G. et al, J Cell Biol 175(6), 2006).

Reviewer's point 5. It is unclear from the text what mechanism leads to the increase of cytosolic IP3 in Figure EV3A.
Response. We believe that cytosolic IP3 increases as a secondary effect of the rise in cytosolic Ca^{2+} levels after addition of the peptide. Indeed, Ca^{2+} enhances PLC activity, which generates IP3 (see for example Horowitz LF et al., J Gen Physiol 2005), and whenever we dampened cytosolic Ca^{2+} rises (e.g. by BAPTA-AM in Figure EV3A or by Xestospongin-C in Figure 2B), we substantially reduced IP3 generation. This point was briefly discussed in the Results section, we will now better explain it.

Reviewer's point 6. A test of xestospongin C under resting conditions is not insightful and does not mitigate concerns based on multiple publications with this reagent. These can be found in many papers outlining its shortcomings (especially the paper by de Smet and Parys 1999 and the recent study by Saleem and Taylor from 2014). The inhibition of SERCA concomitant with IP3R inhibition as shown in the response to my previous concern will not lead to a change in ER Ca2+. Instead, a better control would be within the setup of experiment 2A.
Response. In the first round of revision, the referee was concerned about the possibility that "decreased loading of the ER could account for all observed effects" as "Xestospongin C also inhibits SERCA". In our first response to this point, we included additional data indicating that is not the case, because we observed similar resting ER [Ca^{2+}] between control and XeC-treated cells. This said, we agree that a concomitant inhibition of IP3R and SERCA would not change ER [Ca^{2+}].

However, it is very important to highlight here that, in the presence of equal ER Ca^{2+} levels, if SERCA activity is inhibited, we would expect an increased activity of our peptide, with an increased net Ca^{2+} transfer to mitochondria because of the reduced Ca^{2+} re-uptake within the ER by SERCA. Instead, we measured the exact opposite, a lower one (Figure 2D). This is why we think that in our experimental setup the predominant Xestospongin-C effect is IP3R inhibition. Note that we pretreated cells with 5 μM Xestospongin-C, below the 10-100 μM range observed to inhibit SERCA in the paper cyted by the referee (de Smet and Parys 1999). This reference has been added in the revised manuscript.

Reviewer's point 7. The claim that HK2 is induced as per Figure 4B is inadmissible without a control. The remainder of Figure 4 is compromised due to the lack of comparison between high and low expressing cells, as well as the lack of comparison between high and low HK2 association with MAMs.
Response. We thank the reviewer for this comment, as we agree that the term "induced" is erroneous here and we have removed it from the text. We observe a very low level of HK2 protein levels in lymph node B cells from healthy individuals (Figure 4A, which is representative of at least 6 people, as per standard of the Pathology Unit; we have now included a more representative picture), while there is a certain degree of heterogeneity in B-CLL patients (Figure 4B). However, all patients display a very high and rapid response to the peptide (Figure 4C-D). This indicates that there is not a linear correlation between HK2 levels and peptide effects (see also the low peptide efficacy in non-tumor cells expressing HK2, Figure EV4M, EV4O, EV4P). We think that peptide efficacy depends on the degree of HK2 association to MAMs, but to assess this in fresh cell isolates from B-CLL patients is extremely challenging.

Reviewer's point 8. The analysis of tumor growth for 3 days as done in Figure 5 is not insightful. Standard experimentation for solid tumors uses longer time frames than this. This unusual approaches, especially in the light of "normalization of growth to 100%" (how much did the volumes actually increase in this very short time frame???) raises serious questions.
Response. We consider these in vivo analyses as pilot experiments for future pre-clinical and hopefully clinical developments of our peptide. Indeed, as already mentioned in our previous round of revision, a complex work is needed to adjust the quantity and mode of administration of the peptide, to assess its pharmacodynamic and pharmacokinetic properties and to modulate its amino acid composition in order to maximize its effectiveness towards specific tumor types (e.g. by adapting the cleavable sequence to the proteases found in the extracellular matrix of a certain tumor). This work is under way, and we think it exceeds the scope of the present manuscript. Here, we decided to focus on two unrelated cancer types (breast and colon), which we chose among the most common for obvious reasons of perspective interest, by analyzing the effect of the peptide under conditions of maximal neoplastic growth. Indeed, the rate of doubling of the tumor mass is much higher than that of any human neoplasm (between 48 and 72 hours, Figure 5G-H). The background thinking was that any effect shown by the peptide under these conditions could be translated with increased efficacy in real oncological situations. Of course, the pitfall of this approach, which we are aware is not "standard experimentation", is that we cannot prolong too much the treatment, as we rapidly reach humane endpoints for animals loaded with large and often ulcerating tumor masses. However, we must underline again that peptide treatment had a major impact on neoplastic growth (Figure 5G-H). Tumor volume was expressed not only as a percentage of growth, but also in absolute terms (see the ultrasound inspection of Figure 5G, and the whole Figure 5F). We have now added the kinetic analysis of tumor volume changes in absolute terms (Figure EV5C-D).

Accepted 2 April 2020

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

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Manuscript Number: EMBOR-2019-49117V3

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We performed pilot experiments to estimate sample size in order to maximize differences and detect a pre-specified effect size. Moreover, we considered the sample size according to similar studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We estimated sample size of animal experiments according to similar published studies
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data or animals were excluded from the analyses of the experiments
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.	Animals were randomized after cell implantation
For animal studies, include a statement about randomization even if no randomization was used.	We randomized animals
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.	Data analysis was performed in blind
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding were applied for mice injections, but immuno-hystological analysis were performed in blind
5. For every figure, are statistical tests justified as appropriate?	For each figure we have utilized the appropriate statistic testing, which is justified and explained in the legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used Kolmogoov-Smirnov and Shapiro-Wilk tests to assess if samples display normal distribution and data meet a normal distribution

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Is there an estimate of variation within each group of data?	Yes we estimated appropriate variation (standard deviation or standard error of the mean) in each group of data
Is the variance similar between the groups that are being statistically compared?	Yes the variance is similar between compared groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provided catalog numbers for each antibody in supplementary information
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We included in supplementary information cell lines sources. In our lab, we test all cell lines in culture for mycoplasma contamination every two months. All cell lines used for the project were mycoplasma free.

* 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.	Balb/C wt mice were purchased by ENVIGO and housed in our animal facility following all current guidelines and supervised by a veterinary.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Italian Health Ministry approved our animal experiments on 30-05-2016 n. 547/2016-PR
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.	We confirm compliance with these guidelines, which are also required by the Italian Health Ministry

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study was authorized by the Padua Ethics Committee (Approve Code: n. 3529/AO/14)
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.	This investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki. Written informed consent has been provided to all CLL patients included in the study
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No patients photos were included in our study
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A