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AIF Inhibits Tumor Metastasis by Protecting PTEN from Oxidation

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(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.)

Editor: Esther Schnapp

1st Editorial Decision 13 May 2015

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports and cross-comments that are copied below.

As you will see, all referees agree that the findings are potentially novel and interesting. However, they raise a list of points and concerns that relate to different aspects of the study and indicate that none of the parts goes into sufficient detail. The list is rather long, and ideally all points should be addressed, but most important are the common concerns. These are the intracellular localizations of PTEN and AIF, whether AIF inhibits Wnt signaling through PTEN, that key results should be repeated in a different cell line, and whether AIF controls tumor growth through its effect on PTEN, as the title indicates. Referee 1 also mentions that the effect of AIF on PTEN oxidation should be investigated under oxidative stress. All minor concerns and suggested controls should also be addressed and included.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. 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.

Given that the manuscript has more than 5 main figures, we would publish it as a long article and not a short report. For articles the Results and Discussion sections should be separate, and all materials and methods should be included in the main manuscript file. There are no length limitations on articles.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, along with the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

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.

REFEREE REPORTS:

Referee #1:

This study by Shen et al. aims to describe a role for AIF in controlling PTEN activity through protecting PTEN from inactivation by oxidation.

This is a very interesting study including many exciting data, which at times however made it difficult to follow, because there are some gaps. However, the topic is novel and very interesting, especially from a clinical point of view and should definitely be further evaluated.

Especially from a redox-point of view, the mechanisms described here need to be further evaluated.

Some general concerns:
1) In experiment showing that AIF protects PTEN from oxidation no oxidation is actually induced. This is a problem because there is not experiment demonstrating that AIF is affecting PTEN through simply conformational changes, which under oxidative stress may be irrelevant.
2) While the observation of short AIF partial proteins and their effect on PTEN are clear, how does short AIF compare to AIF full length in effects on PTEN?
3) is the interaction between AIF and PTEN of covalent nature? it would be helpful to see the full membrane in supplemental fig 1b to further elaborate on this. Also, why is there a prominent band in the H2O2 untreated sample around 70 kda and why is there no oxidized PTEN in samples where no AIF was added?
4) It is not clear to the reviewer why the PTENC71S mutant was used as control. Is there a suspicion that this cysteine plays a role in the AIF interaction? is this PTEN mutant binding to AIF?
5) If H2O2 induces AIF nuclear localization, how can it then protect mitochondria PTEN from oxidation? Is AIF also binding nuclear PTEN?

Minor concerns:
1) some results are over-interpreted: page 8, line 11; from a reviewers perspective: the anti-oxidant effect of AIF on PTEN has not been demonstrated sufficiently. OR Figure 5: the evidence that AIF loss activated WNT is there but the evidence that this occurs through PTEN regulation is missing. For example: shRNA PTEN in combination with shRNA AIF should be included and compared to single shRNA PTEN and single shRNA AIF.
2) "by the way" should not be used.
3) Fig.3D should have PTENC71S and C124S as control
4) Fig. 3F is mislabeled: non-reducing and reducing is switched.
5) Throughout Fig 3 and the corresponding supplemental figures, exogenous stress induction to study the redox-component is clearly needed.
6) In S1F: there is no PTEN oxidation observed under steady state conditions, however, once AIF is
knocked down, PTEN oxidation is increased. According to the data shown, loss of AIF does not induce ROS. How is that to be explained? Does AIF regulate mitochondrial ROS? DCF measures peroxides. Is superoxide regulated instead?

7) AIF::PTEN binding needs to be evaluated under oxidative stress

Referee #2:

Shen et al report the physical and functional interaction between the oxidoreductase apoptosis-inducing factor (AIF) and the tumor suppressor phosphatase PTEN. It is shown that AIF and PTEN directly interact through the phosphatase domain of PTEN and the oxidoreductase domain of AIF. This interaction resulted in decreased PTEN oxidation and increased PTEN phosphatase activity, both in vitro and in cells, and a fraction of PTEN was found to co-localize with AIF inside mitochondria. It is further shown that AIF knockdown triggers an EMT program in a PTEN oxidation dependent manner. Finally, it is reported that AIF loss promotes tumor metastasis in xenograft models, as well as a correlation of low AIF expression and poor outcome in colon cancer. The findings are sound and important in the field, and provide good evidence of a tumor suppressor role for AIF through its positive redox regulation of PTEN activity.

Specific comments:

1. As mentioned in the discussion, the reported intra-mitochondrial localization of PTEN is in contrast with previous reports showing PTEN localization to the mitochondrial surface (Zu et al. 2011; Liang et al., 2014; the authors should incorporate the work by Zhou et al. 2006 PMID: 16502258). Since PTEN lacks a canonical MLS sequence, a possibility is that PTEN is distributed in several mitochondria locations and that AIF "transports" a fraction of PTEN to the mitochondria interior. Subcellular distribution experiments using recombinant PTEN and AIF that bind or not bind to each other could be informative at this respect.

2. Detailed methodological information should be provided on the mitochondrial targeting sequence fused to PTEN to construct mito-PTEN.

3. Some references in the text are not the appropriate ones:
   - page 10, line 4: Papa et al 2014 should be changed by one of the original references using the C124S mutant (such as Maehama et al 1998 PMID: 9593664; or Myers et al 1998 PMID: 9811831).
   - page 15, last line: Wang et al 2011 should be changed by the appropriate reference: Huang et al 2012 PMID: 23012657.

4. Other small corrections/comments:
   - page 16: PTEN-Long is defined as "a translational variant of PTEN that adds 173 N-terminal amino acids to the canonical protein"; PTENalpha is defined as "an N-terminally extended form of PTEN". PTEN-Long and PTENalpha have identical amino acid sequence, including the 173 N-terminal amino acids, but they were named differently. The text should be changed to avoid ambiguity.
   - page 17, line 2: please change "oxidation of partial PTEN" by "partial oxidation of PTEN"

Referee #3:

Comments on manuscript number: EMBOR-2015-40536-T "AIF Governs Tumor Metastasis by Protecting PTEN from Oxidation" by Shao-Ming Shen and co-authors

In their study Shao-Ming Shen and colleagues investigate a potential role of AIF in tumorigenesis. Through an interactor screen they identify the tumor suppressor PTEN as a novel interaction partner of AIF. Subsequently, they show that AIF protects PTEN against inhibitory oxidation. By maintaining PTEN in its active state, AIF functions as a negative regulator of PI3K signaling. Furthermore, depletion of AIF increases cell motility and invasiveness in vitro and promotes metastasis in xenotransplantation experiments in vivo. Previously, AIF was known as a multifunctional protein with pro-apoptotic and pro-survival functions. The new findings by Shen and colleagues that link AIF to tumor suppressive signaling processes via PTEN, are very interesting and broadly relevant. This new and exciting functional interaction between AIF and PTEN and the
role of PTEN activity downstream of AIF is well documented and in most cases supported by multiple independent lines of experimentation. For example, the AIF-PTEN interaction is analyzed both by immunoprecipitation and by GST-pull down in vitro, AIF knockdown experiments are combined with rescue by different variants of AIF, and it is nicely demonstrated that PTEN is epistatic to AIF by use of a non-oxidizable PTEN mutant. However, I am not convinced by the mechanistic explanation for the anti-metastatic effect of AIF where the authors claim that depletion of AIF triggers elevated β-catenin signaling and thereby leads to pro-metastatic epithelial-mesenchymal transitions through upregulation of ZEB1.

Specific comments:
1. A major concern is the fact that key experiments were carried out almost exclusively with a single cell line, SW620. SW480 and the related SW620 have high β-catenin activity, show nuclear β-catenin, express comparatively low or no E-cadherin but high levels of ZEB1 (and other regulators of EMT) and have at least a partially mesenchymal character which may be reflected in the heterogeneous appearance of these cells that present with different morphologies in culture (see for example Buck et al 2007, Mol Cancer Ther 6, 532). This could explain why some of the observed effects upon AIF knockdown are rather minor. On the other hand, SW620 cells may already be pre-sensitized to AIF-dependent changes in intracellular signaling, leading to an overestimation of the potency of AIF as a regulator of tumorigenesis and metastasis. Therefore, it is of vital importance to corroborate the findings made with SW620 cells by experiments with other cell lines from different tumor entities that have no Wnt pathway activating mutations.
2. Urbano and colleagues (Urbano et al. 2005, EMBO J 24, 2815) completely knocked out AIF in two colorectal cancer cell lines and found that AIF knockout cells failed to form tumors in athymic mice which contrasts with the consequences of AIF knockdown in SW620 cells. The authors should cite the work by Urbano et al and comment on the apparent discrepancy in the Discussion.
3. Figure 1 could be simplified by bringing together the domain mapping studies shown in panels K and O. It also appears to me that the results of these experiments are not entirely conclusive. There could be two binding sites for PTEN in AIF that colocalize with the FAD binding domains. Furthermore, the amount of the GST-AIF-N bait proteins needs to be brought to the same level as the other GST-fusions otherwise a weak interaction might be obscured.
4. According to earlier work (ref. Delettre et al., 2006b) the AIFsh3 isoform is not expressed in most cells and tissues. Shen and colleagues seem to be able to detect AIFsh3 in SW620 cells. However, the antibody used also reacts with full-length AIF and Figure 1L clearly shows a cropped and reassembled image. I suggest showing a Western blot that displays AIF and its isoforms (or degradation products) in a single image to be able to judge the relative amounts of AIF and its isoforms. Furthermore, because of antibody reactivity with multiple forms of AIF, AIFsh3 expression must be confirmed on mRNA level.
5. AIF localizes to mitochondria and the authors hypothesize that AIF directly interacts with and acts upon PTEN. It thus becomes important for the authors to show that also PTEN can be found in mitochondria. In fact, the mitochondrial localization of PTEN was known before and was not newly discovered in the current study. From this point of view the data presented in Figure 2 appear excessive. An issue more important than the occurrence of PTEN in mitochondria is the question whether mitochondrial PTEN can signal to AKT and is relevant for the AIF knockdown phenotype. This is not sufficiently demonstrated by Figure 3A,B. In this regard it is curious that AIF knockdown phenotypes can be rescued by expression of AIFsh3 which does not have a mitochondrial localization signal and is cytoplasmic. Would the mitochondrial AIFsh2 also rescue? In light of work by Naguib and coworkers (Nagib et al 2015, Mol Cell 58, 255, "PTEN Functions by Recruitment to Cytoplasmic Vesicles") the authors should check sedimentation profiles of other organelle markers in Figure 2A,B and address in their Discussion where in the cell AIF or one of its isoforms interacts with PTEN and how this is related to the site of action of PTEN.
6. I am puzzled by the large excess of AIF over PTEN that is required to induce comparably small effects on PTEN oxidation and PTEN enzymatic activity as shown in Figure 3D,E. Is PTEN such a poor substrate or is it that the AIF effects on PTEN are non-enzymatic? Can the authors comment on this?
7. Based on the results of the current study one would assume that tumor cells gain a growth advantage by loss of AIF. Hence, AIF should frequently be downregulated or mutated in tumor cells. Yet, previous work (not cited by the authors: Jeong et al 2006, APMIS 144, 867) showed that about 80% of colorectal tumors have elevated AIF expression. Also in the Chinese colorectal cancer patient cohort studied by Shen and colleagues the majority of cases has high AIF expression and differences in overall survival depending upon the AIF status barely reach statistical significance.
(Figure 7M). AIF is a multifunctional protein and perhaps its pro-survival function is of greater importance in tumorigenesis than its role as antagonist of PI3K signaling. Thus, it could be informative to stratify patient cohorts according to the presence of PI3K mutations and then see how AIF expression relates to prognosis. The authors need to cite earlier work and address these issues in their discussion.

8. In previous studies Prdx1 and Txnip were implicated in control of PTEN activity by reversible oxidation. Do these factors act on PTEN independently of AIF or is there a connection? The authors should address the issue of multiple independent PTEN control mechanisms or their potential interconnection in the Discussion.

9. The authors try to support their hypothesis that AIF counteracts EMT by GSEA using TCGA transcriptome data. Recent reports show that colorectal tumor preparations can be contaminated by stromal cells and that "EMT" signature gene expression in fact is due to the stromal contaminants (Kim and Verhaak, 2015 Nat Genetics, 47, 307). Caution is warranted when using bulk tumor transcriptome data to support gene expression changes in epithelium-derived tumor cells specifically. Immunohistochemical analyses of AIF and EMT marker coexpression in colorectal tumors could be an alternative.

10. In Figure 6A changes in ZEB1, VIM and FN1 expression are negligible on RNA level, but clearly seen in Western blot analyses. Furthermore, the authors claim that ZEB1 upregulation is a consequence of Wnt/β-catenin pathway activation upon knockdown of AIF and suggest that ZEB1 expression is responsible for the induction of EMT. However ZEB1 is not an exclusive target of Wnt signaling and ZEB1 is not the only inducer of EMT. Therefore, the authors should also examine the expression of SNAIL1, SLUG and TWIST1 and especially of miR200 family members. Downregulation of the latter could readily explain increased ZEB1 protein levels. Importantly, since the authors claim that AIF governs EMT and metastasis of cancer cells through Wnt/β-catenin pathway activation they need to demonstrate that further upregulation of the already high levels of ZEB1 and the further promotion of EMT in their system indeed depends upon β-catenin/TCF activity. Otherwise, the relevant passages in the manuscript need to be rephrased for the fact that the authors’ claims are merely based on correlations.

11. Likewise, none of the genes examined in Figures 4 and 5 are under sole control of Wnt signaling not even AXIN2 (see for example Morris et al 2008, Nature 455, 552; Hughes and Brady, 2005, Exp Cell Res 303, 32). To substantiate their claims the authors need to provide clear proof that the observed alterations in gene expression are due to changes in β-catenin/TCF activity.

12. The authors implicate β-catenin signaling in the phenotypic consequences of AIF knockdown because of increased AKT activity and the ensuing inhibitory phosphorylation of GSK3. Although this is a widely held belief, the architecture of the Wnt/β-catenin pathway actually prohibits such crosstalk (Ng et al, 2009 J Biol Chem. 284, 35308 "Phosphatidylinositol 3-kinase signaling does not activate the Wnt cascade"). In fact, there could be alternative mechanisms to activate β-catenin downstream of PTEN and the observed effects of AIF knockdown could be completely independent of β-catenin signaling (see for example Liliental et al, 2000, Curr Biol 10, 401; Myant et al 2013, Cell Stem Cell 12, 761). Therefore, to be able to maintain their mechanistic explanations the authors need to firmly establish β-catenin-dependency of the observed phenotypic changes in response to AIF depletion.

13. In Figures 4 and 5 the authors analyze the expression of a set of six presumptive Wnt/β-catenin target genes. However, sometimes one or more of the genes are omitted from the analyses (AXIN2, LEF1, MMP7; Figures 4G, 5A, 5C). I suggest to consistently show the data for all six genes. Moreover, since some of the expression changes observed are very small on RNA level (MMP7, AXIN2, ID2 in Figures 4E-G and 5D-F), irrespective of their statistical significance, I recommend to express the confirmation changes by Western blotting.

14. In many instances, the authors nicely substantiate their work by rescue experiments with different AIF constructs or oxidation-resistant PTEN. However, they deviate from this practice in Figure 7. To further support the relevance of the newly discovered AIF-PTEN axis in EMT and metastasis, at least the impact of combined AIF depletion and PTEN-C71S expression in the migration/invasion studies and, importantly, in xenotransplants should be shown. Can PTEN-C71S revert the cellular phenotypes of AIF loss-of-function? An informative extension of this would be to deplete both AIF and ZEB1 in these assays to demonstrate that ZEB1 is the critical factor downstream of AIF and PTEN.

15. In Figure 7I, HT29 cells are used as a second model to study the impact of AIF knockdown on tumor formation and metastasis but only efficacy of the AIF knockdown is shown. Does reduction of AIF levels have the same functional consequences on PTEN activity, phosphorylation of AKT and GSK, and the expression of Wnt target genes or EMT markers as in SW620?
16. The y-axes in Figure 7H and L are labeled identically but it is not clear whether the same thing is shown by the diamonds in both graphs, the number of mice with liver metastases or the number of metastatic nodules per liver. Furthermore, in Figure 7O it should be specified whether lymph node or distant organ metastasis was considered because the two have different prognostic values.

17. Most of the figures are overcrowded and the lettering and lines are almost too faint. I am afraid that upon the necessary size reduction the figures will no longer be legible. The figures should be given a thorough work over to improve their quality and to eliminate some apparent errors. For example, in contrast to the statement in the legend, Figure 2E does not show SW620 cells but mitochondria. In Figures 2C, 2F, 5E and 5F positions of the side labels shifted, „Non-reducing” and „Reducing” were mixed up, and the y-axis labels are incorrectly positioned, respectively. In Figure 5A the PTEN low and high status can only be guessed. Abbreviations in Figure 1J need to be explained in the legend. For the GSEA in Figure 4, panels H and I normalized enrichment scores (NES) and p-Values need to be shown (just as was done for Figure 6E). In both cases (Figures 4H,I and 6E) it should be indicated what is represented by the red/blue scale bars.

18. Similarly, Figures S1 and S2 are very small and hardly legible when printed in their original size. Figure size and lettering need to be increased.

19. I think there is a certain degree of redundancy provided by Figure 3 panels F/G, and I/L. The consequences of AIF knockdown and rescue could easily be put together without loss of clarity.

20. Likewise, in Figure 4, the number of panels could be reduced by combining panels D with G (AIF knockdown and rescue) and panels E with F (overexpression of various AIF constructs).

21. In Figure 7F serial sections should be shown and the tumor area be marked, especially in the E-cadherin-negative case.

22. What is the reason for showing Figure 7G? It seems to me that it has no information value.

23. SW480 cells are mentioned in the Materials and Methods section but they are not used in the study.

24. The authors do not provide a description of their construct targeting PTEN to mitochondria.

25. Several parts of the Materials and Methods sections are identical between the main manuscript and the Expanded View Content. These are unnecessary duplications and increase main manuscript length.

26. Figure S1H: Knockdown of AIF appears to have different effects on AKT and GSK3 phosphorylation (increase, decrease and neutral) in different cells which cannot simply be explained by the PTEN status. The authors should quantify changes in pAKT and pGSK3 in DU145, PC-3 and LNCaP cells and comment on the possibility that additional factors impinge on the functional connections between AIF, PTEN, AKT and GSK3.

27. Table S2 does not provide sufficient data to allow evaluating the significance of the LC-MS/MS results. Furthermore, the initial interactor screen and the LC-MS/MS related methodology are not described in the manuscript. On the other hand, Table S2 also does not provide information that would be essential for the understanding of the study. Therefore, I suggest to either omit Table S2 or to provide additional information to make its inclusion worthwhile, including a detailed description of the experimental procedures. For example, what was the number of peptides found, the sequence coverage and significance scores, etc.? Moreover, the candidates could be sorted based on their intracellular localization.

Detailed response to referees’ comments

Referee #1:

This study by Shen et al. aims to describe a role for AIF in controlling PTEN activity through protecting PTEN from inactivation by oxidation. This is a very interesting study including many exciting data, which at times however made it difficult to follow, because there are some gaps.

However, the topic is novel and very interesting, especially from a clinical point of view and should
definitely be further evaluated. Especially from a redox-point of view, the mechanisms described here need to be further evaluated.

**RESPONSE:** Thanks for the reviewer’s enthusiasm on this study. We have revised the manuscript according to your concerns, especially the related mechanisms have been further evaluated.

**General concern 1:** In experiment showing that AIF protects PTEN from oxidation no oxidation is actually induced. This is a problem because there is not experiment demonstrating that AIF is affecting PTEN through simply conformational changes, which under oxidative stress may be irrelevant. In addition to this: a AIF catalytically dead mutant needs to be involved as control for mechanism.

**RESPONSE:** Thanks for the suggestion. As we described in the revised version, a key hallmark of cancer cells is unrestrained growth. Because ROS generation is a byproduct of cell growth, cancer cells sustain a much higher level of ROS production compared to normal cells, and thus it was believed that cancer cells are characterized with high status of oxidative stress(Harris et al, 2015; Trachootham et al, 2009) (see lines 9 -13 of page 9). Therefore, our results revealed that the endogenous oxidative stress induces PTEN oxidation in cancer cells with AIF knockdown. Considering the reviewer’s concern, we also tested the role of AIF on PTEN oxidation under the treatment of H$_2$O$_2$, and the result showed that the exogenous H$_2$O$_2$-induced PTEN oxidation could also be significantly enhanced by AIF knockdown (current Fig S2I/J), and the related description is provided in the lines 10-12 of page 11 of the revised version. In addition, oxidoreductase domain-deleted AIFsh (Delettre et al, 2006b) was used as control. Indeed, the isoform failed to rescue PTEN oxidative modification and Akt activation induced by AIF knockdown (current Fig 3H).

**General concern 2:** While the observation of short AIF partial proteins and their effect on PTEN are clear, how does short AIF compare to AIF full length in effects on PTEN?

**RESPONSE:** A good comment. In the revised version, we showed that AIFsh2 and AIFsh3 but not AIFsh reversed the role of AIF knockdown on PTEN oxidation and Akt phosphorylation to a similar extent as full-length AIF (see current Fig 3H).

**General concern 3:** Is the interaction between AIF and PTEN of covalent nature? It would be helpful to see the full membrane in supplemental Fig 1b to further elaborate on this. Also,
why is there a prominent band in the H$_2$O$_2$ untreated sample around 70 kDa and why is there no oxidized PTEN in samples where no AIF was added?

**RESPONSE:** We show the full membrane in the current Figure S2B (previous Figure S1B). According to this, we proposed that the interaction between AIF and PTEN is not of covalent nature, because co-existence of PTEN did not result in a ~120 kDa band (67 kDa AIF and 55 kDa PTEN) when detected with either anti-PTEN or anti-AIF antibody. With the full membrane, we can also show that, in H$_2$O$_2$ untreated samples, PTEN was oxidized under air to different extents including the band around 70 kDa, the latter being disappeared with the existence of high molecular weight form of oxidized PTEN (on the top of the membrane) under H$_2$O$_2$ exposure, suggesting that H$_2$O$_2$ treatment caused a complete and uniform oxidation of PTEN. When AIF was added, the reductive PTEN appeared in the absence and presence of H$_2$O$_2$, further indicating the anti-oxidant role of AIF on PTEN. The related description has been given in the line 21 of page 9 to the first line of page 10 in the revised version.

**General concern 4:** It is not clear to the reviewer why the PTEN-C71S mutant was used as control. Is there a suspicion that this cysteine plays a role in the AIF interaction? Is this PTEN mutant binding to AIF?

**RESPONSE:** As we pointed out (see lines 2-5 of page 10 and line 8-11 of page 12), it was reported that H$_2$O$_2$-mediated PTEN oxidation resulted in an intra-molecular disulfide bond formed between Cys$^{124}$ and Cys$^{71}$. Mutation of either site resists oxidative modification by H$_2$O$_2$ (Lee et al, 2002). On the other hand, the C71S mutant still keeps the partial activity of PTEN relative to its wild-type form (Greiner et al, 2013). Therefore, we used the PTEN-C71S mutant as an effective tool to test whether all the downstream effects of AIF knockdown were mediated by PTEN oxidation. Actually, we showed that the PTEN-C71S mutant still interacts with AIF (the current Fig S2N), indicating that this cysteine does not contribute to the PTEN-AIF interaction.

**General concern 5:** If H$_2$O$_2$ induces AIF nuclear localization, how can it then protect mitochondria PTEN from oxidation? Is AIF also binding nuclear PTEN?

**RESPONSE:** We used lower concentration (100µM) of H$_2$O$_2$, which did not induce AIF nuclear localization, as shown in current Fig S2K.
Minor concerns 1: Some results are over-interpreted: page 8, line 11; from a reviewer’s perspective: the anti-oxidant effect of AIF on PTEN has not been demonstrated sufficiently. OR Figure 5: the evidence that AIF loss activated WNT is there but the evidence that this occurs through PTEN regulation is missing. For example: shRNA PTEN in combination with shRNA AIF should be included and compared to single shRNA PTEN and single shRNA AIF.

RESPONSE: We have seriously revised our statement. For example, the previous phrase “suggesting the anti-oxidative effect of AIF on PTEN” has been deleted in the revised version. Of note, we actually showed the results of shRNA PTEN in combination with shRNA AIF and their comparison to single shRNA PTEN and single shRNA AIF. Our results showed that AIF or PTEN silencing increased expressions of WNT/β-catenin signaling target genes (current Fig 5B). However, knockdown of AIF in combination with PTEN silencing did not further enhance the expressions of these genes (current Fig 5B). These results combined with the data from the PI3K/Akt inhibitor LY294002 (current Fig 5C) and NAC treatment (current Fig 5D) should support that PTEN oxidative inactivation mediates AIF knockdown-activating WNT/β-catenin signaling.

Minor concern 2: "by the way" should not be used.

RESPONSE: Thanks, we have deleted the phrase throughout the manuscript.

Minor concern 3: Fig.3D should have PTEN-C71S and C124S as control

RESPONSE: We have added PTEN-C124S mutant as a control in the re-tested Fig 3D.

Minor concern 4: Fig. 3F is mislabeled: non-reducing and reducing is switched.

RESPONSE: Sorry for this mislabel, which has been corrected in this revised version.

Minor concern 5: Throughout Fig 3 and the corresponding supplemental figures, exogenous stress induction to study the redox-component is clearly needed.

RESPONSE: As we responded to the reviewer’s general concern 1, cancer cells are characterized with high status of oxidative stress. Therefore, our results revealed that the endogenous oxidative stress induces PTEN oxidation especially in cancer cells with AIF knockdown. Considering the reviewer’s concern, we also showed that the exogenous H2O2-induced
PTEN oxidation could be significantly enhanced by AIF knockdown in the current Fig S2I/J.

**Minor concern 6:** In S1F: there is no PTEN oxidation observed under steady state conditions, however, once AIF is knocked down, PTEN oxidation is increased. According to the data shown, loss of AIF does not induce ROS. How is that to be explained? Does AIF regulate mitochondrial ROS? DCF measures peroxides. Is superoxide regulated instead?

**RESPONSE:** We think that AIF can prevent PTEN oxidation as an oxidoreductase under steady state conditions, although cancer cells are under oxidative stress. When AIF is deficient, such a preventing effect is also lost. Therefore, PTEN oxidation is increased, although loss of AIF does not increase ROS. These explanations could also be supported by the result showing that AIF knockdown also increased PTEN oxidation induced by H₂O₂ exposure (current Fig S2I/J).

**Minor concern 7:** AIF: PTEN binding needs to be evaluated under oxidative stress

**RESPONSE:** In the current Fig S2L, we showed that AIF still binds PTEN under H₂O₂ exposure.

**Referee #2:**

Shen et al report the physical and functional interaction between the oxidoreductase apoptosis-inducing factor (AIF) and the tumor suppressor phosphatase PTEN. It is shown that AIF and PTEN directly interact through the phosphatase domain of PTEN and the oxidoreductase domain of AIF. This interaction resulted in decreased PTEN oxidation and increased PTEN phosphatase activity, both in vitro and in cells, and a fraction of PTEN was found to co-localize with AIF inside mitochondria. It is further shown that AIF knockdown triggers an EMT program in a PTEN oxidation dependent-manner. Finally, it is reported that AIF loss promotes tumor metastasis in xenograft models, as well as a correlation of low AIF expression and poor outcome in colon cancer.

**The findings are sound and important in the field, and provide good evidence of a tumor suppressor role for AIF through its positive redox regulation of PTEN activity.**

**RESPONSE:** Thanks for the reviewer’s interest and positive evaluation.

**Specific comment 1:** As mentioned in the discussion, the reported intra-mitochondrial localization of PTEN is in contrast with previous reports showing PTEN localization to the mitochondrial surface (Zu et al. 2011; Liang et al., 2014; the authors should incorporate
the work by Zhou et al. 2006 PMID: 16502258). Since PTEN lacks a canonical MLS sequence, a possibility is that PTEN is distributed in several mitochondria locations and that AIF "transports" a fraction of PTEN to the mitochondria interior. Subcellular distribution experiments using recombinant PTEN and AIF that bind or not bind to each other could be informative at this respect.

**RESPONSE:** We have incorporated the work by Zhu Y et al and cited the paper (Zhu Y, 2006). Indeed, we found that AIF-interacting PTEN-N fragment was more potently localized into mitochondria than PTEN-C fragment, which did not bind with AIF, as shown below (T, total; C, cytosol; N, nuclei; M, mitochondrion). According to your good comment, we revised the related discussion (lines 11-12 of page 20) as follows: A possibility is that PTEN is distributed in several mitochondria locations and that AIF carries a fraction of PTEN to the mitochondria interior, which could be partially supported by the fact that AIF-interacting PTEN-N was more potently localized into mitochondria than PTEN-C, which did not bind with AIF.

![Subcellular distribution experiments using recombinant PTEN and AIF](image)

**Specific comment 2:** Detailed methodological information should be provided on the mitochondrial targeting sequence fused to PTEN to construct mito-PTEN.

**RESPONSE:** We have described the construction of mito-PTEN in the revised MATERIALS AND METHODS section (line 21 of page 23 of page 24).

**Specific comment 3:** Some references in the text are not the appropriate ones:

- page 10, line 4: Papa et al 2014 should be changed by one of the original references using the C124S mutant (such as Maehama et al 1998 PMID: 9593664; or Myers et al 1998 PMID: 9811831).

- page 15, last line: Wang et al 2011 should be changed by the appropriate reference: Huang et al 2012 PMID: 23012657.

**RESPONSE:** We have changed these references (see line 9-10 of page 12 and line 15-16 of page 19) in the revised version. Of note, authors of the paper with PMID: 23012657 should be Putz et al.
Specific comment 4. Other small corrections/comments:
- page 16: PTEN-Long is defined as "a translational variant of PTEN that adds 173 N-terminal amino acids to the canonical protein"; PTENalpha is defined as "an N-terminally extended form of PTEN". PTEN-Long and PTENalpha have identical amino acid sequence, including the 173 N-terminal amino acids, but they were named differently. The text should be changed to avoid ambiguity.
- page 17, line 2: please change "oxidation of partial PTEN" by "partial oxidation of PTEN"

RESPONSE: Thanks, we have done it.

Referee #3:
Comments on manuscript number: EMBOR-2015-40536-T "AIF Governs Tumor Metastasis by Protecting PTEN from Oxidation" by Shao-Ming Shen and co-authors. In their study Shao-Ming Shen and colleagues investigate a potential role of AIF in tumorigenesis. Through an interactor screen they identify the tumor suppressor PTEN as a novel interaction partner of AIF. Subsequently, they show that AIF protects PTEN against inhibitory oxidation. By maintaining PTEN in its active state, AIF functions as a negative regulator of PI3K signaling. Furthermore, depletion of AIF increases cell motility and invasiveness in vitro and promotes metastasis in xenotransplantation experiments in vivo. Previously, AIF was known as a multifunctional protein with pro-apoptotic and pro-survival functions. The new findings by Shen and colleagues that link AIF to tumor suppressive signaling processes via PTEN, are very interesting and broadly relevant. This new and exciting functional interaction between AIF and PTEN and the role of PTEN activity downstream of AIF is well documented and in most cases supported by multiple independent lines of experimentation. For example, the AIF-PTEN interaction is analyzed both by immunoprecipitation and by GST-pull down in vitro, AIF knockdown experiments are combined with rescue by different variants of AIF, and it is nicely demonstrated that PTEN is epistatic to AIF by use of a non-oxidizable PTEN mutant. However, I am not convinced by the mechanistic explanation for the anti-metastatic effect of AIF where the authors claim that depletion of AIF triggers elevated β-catenin signaling and thereby leads to pro-metastatic epithelial-mesenchymal transitions through upregulation of ZEB1.
RESPONSE: Thanks for the reviewer’s good summary and positive evaluation on this study. We have revised the manuscript according to your following concerns, especially the mechanistic explanation for the anti-metastatic effect of AIF.

Specific comment 1: A major concern is the fact that key experiments were carried out almost exclusively with a single cell line, SW620. SW480 and the related SW620 have high β-catenin activity, show nuclear β-catenin, express comparatively low or no E-cadherin but high levels of ZEB1 (and other regulators of EMT) and have at least a partially mesenchymal character which may be reflected in the heterogeneous appearance of these cells that present with different morphologies in culture (see for example Buck et al 2007, Mol Cancer Ther 6, 532). This could explain why some of the observed effects upon AIF knockdown are rather minor. On the other hand, SW620 cells may already be pre-sensitized to AIF-dependent changes in intracellular signaling, leading to an overestimation of the potency of AIF as a regulator of tumorigenesis and metastasis. Therefore, it is of vital importance to corroborate the findings made with SW620 cells by experiments with other cell lines from different tumor entities that have no WNT pathway activating mutations.

RESPONSE: Thanks for this good comment. In the revised version, most of the key experiments made with SW620 cells have been performed in HT29 cells (Fig S2E, Fig 3K, Fig S3A and Fig S4A) and DU145 cells (Fig S2F and Fig S4B), which have not been reported to have WNT pathway activating mutations. Also, we pointed out in lines 8-14 of page 15 that “Considering that some previous reports showed that SW620 cells have at least a partially mesenchymal character, which may be reflected in the heterogeneous appearance of these cells that present with different morphologies in culture (Buck et al, 2007), we also detected the potential EMT-inducing effect of AIF knockdown in other two cancer cell lines HT29 and DU145 cells. The results demonstrated that, like that seen in SW620 cells, AIF knockdown could also trigger these two cell lines to undergo EMT (Figure S4A/B)”.

Specific comment 2: Urbano and colleagues (Urbano et al. 2005, EMBO J 24, 2815) completely knocked out AIF in two colorectal cancer cell lines and found that AIF knockout cells failed to form tumors in athymic mice which contrasts with the consequences of AIF
knockdown in SW620 cells. The authors should cite the work by Urbano et al and comment on the apparent discrepancy in the Discussion.

**RESPONSE:** In the revised discussion (line 23 of page 22 to line 4 of page 23), we have cited the paper, and pointed out that a previous report, which showed that human colon carcinoma cell lines with AIF knockout by homologous recombination failed to form tumors in athymic mice or grow in soft agar (Urbano et al, 2005), which was completely inconsistent with our results from cancer cells with AIF knockdown. In Urbano et al’s report, they completely knockout AIF, while we silenced AIF expression with shRNAs. We extrapolated that the reasons to cause the apparent discrepancy might due to different levels of AIF loss in two different assays, because AIF presents dual roles in cell death and survival.

**Specific comment 3:** Figure 1 could be simplified by bringing together the domain mapping studies shown in panels K and O. It also appears to me that the results of these experiments are not entirely conclusive. There could be two binding sites for PTEN in AIF that colocalize with the FAD binding domains. Furthermore, the amount of the GST-AIF-N bait proteins needs to be brought to the same level as the other GST-fusions otherwise a weak interaction might be obscured.

**RESPONSE:** We combined the domain mapping studies into one single panel in the revised version (current Fig 1K). We also further detected the potential interaction of PTEN-N with FAD1 and/or FAD2 domains-deleted AIF mutant. The results showed that FAD1 or FAD2-deleted AIF mutant still interacted with PTEN-N, which was completely lost when both FADs were deleted (Fig 1K, right panel), supporting that two FAD domains of AIF are required for its interaction with PTEN. The related data were also be described in lines 11-17 of page 7 in the revised version. Furthermore, because the amount of the GST-AIF-N bait proteins was not brought to the same level as the other GST-fusions, we deleted the previous statement that GST-AIF-N had a weak binding with PTEN-N.

**Specific comment 4:** According to earlier work (ref. Delettre et al., 2006b) the AIFsh3 isoform is not expressed in most cells and tissues. Shen and colleagues seem to be able to detect AIFsh3 in SW620 cells. However, the antibody used also reacts with full-length AIF and
Figure 1L clearly shows a cropped and reassembled image. I suggest showing a Western blot that displays AIF and its isoforms (or degradation products) in a single image to be able to judge the relative amounts of AIF and its isoforms. Furthermore, because of antibody reactivity with multiple forms of AIF, AIFsh3 expression must be confirmed on mRNA level.

**RESPONSE:** Thank you for the good suggestion. In the revised version, we re-performed the assay of previous Figure 1L, and showed a western blot that displays AIF and two of its isoforms AIFsh2 and AIFsh3 in a single immunoblots with an antibody specifically against residues 151-168 of AIF, which can detect AIFsh2 and AIFsh3 besides AIF-FL, and the results showed that SW620 cells and other three cell lines expressed AIF-FL, AIFsh2 and AIFsh3 to a different degree, with AIFsh3 protein to a lowest degree (current Fig S1B). We also used RT-PCR with specific primers according to a previous report (Delettre et al, 2006b) to confirm the expression of AIFsh, AIFsh2 and AIFsh3 on mRNA level (current Fig S1A). The related description was given in the line 25 of page 6 to line 6 of page 7 of the revised version.

**Specific comment 5:** AIF localizes to mitochondria and the authors hypothesize that AIF directly interacts with and acts upon PTEN. It thus becomes important for the authors to show that also PTEN can be found in mitochondria. In fact, the mitochondrial localization of PTEN was known before and was not newly discovered in the current study. From this point of view the data presented in Figure 2 appear excessive. An issue more important than the occurrence of PTEN in mitochondria is the question whether mitochondrial PTEN can signal to AKT and is relevant for the AIF knockdown phenotype. This is not sufficiently demonstrated by Figure 3A,B. In this regard it is curious that AIF knockdown phenotypes can be rescued by expression of AIFsh3 which does not have a mitochondrial localization signal and is cytoplasmic. Would the mitochondrial AIFsh2 also rescue? In light of work by Naguib and coworkers (Naguib et al 2015, Mol Cell 58, 255, "PTEN Functions by Recruitment to Cytoplasmic Vesicles") the authors should check sedimentation profiles of other organelle markers in Figure 2A,B and address in their Discussion where in the cell AIF or one of its isoforms interacts with PTEN and how this is related to the site of action of PTEN.
RESPONSE: Although several lines of evidence recently demonstrated the PTEN localization to the mitochondria (Bononi et al, 2013; Zhu et al, 2006; Zu et al, 2011), and PTEN-Long was also be shown to localize to the cytoplasm and the mitochondria (Liang et al, 2014), but Pagliarini et al’s report from a mass spectrometry, GFP tagging, and machine learning-based analysis did not report PTEN in a mitochondrial compendium (Pagliarini et al, 2008). Actually, localization of PTEN in mitochondria, especially on the outer membrane of mitochondria or inter-mitochondria, is controversial. Therefore, we also continued to decide whether PTEN is also localized in mitochondria, as we pointed out in the line 23 of page 7 to line 4 of page 8 and line 18 of page 19 to the first line page 20 of this revised version. In our work, we clearly showed that PTEN is also an internal mitochondrial protein by immuno-electron microscopy. Therefore, we think that the data presented in Figure 2 is not excessive, although it was not novel. To this view, we still kept the data into the manuscript.

Our results showed that the ectopically expressed mito-PTEN was capable of significantly inhibiting Akt phosphorylation (Fig 3A/B), suggesting that mitochondrial PTEN can signal to Akt. To consolidate this, we also showed that AIF knockdown phenotypes can be rescued by expression of AIFsh2 as well as full-length AIF which have a mitochondrial localization signal (current Figure 3H). On the other hand, AIF knockdown phenotypes can be rescued by expression of AIFsh3 which is cytoplasmic without a mitochondrial localization signal (Figure 3H). Therefore, we think that either mitochondrial AIF or cytoplasmic short AIF isoform could interfere with PTEN and thus Akt signal. In addition, we also give the following discussion in lines 4-8 of page 20: Recently, Naguib and coworkers reported that cytoplasmic PTEN is distributed along microtubules, tethered to vesicles. It remains to be investigated whether PTEN is brought into mitochondria and other compartments by such vesicles. Actually, the previous reports also showed the localization of PTEN in the endoplasmic reticulum (Bononi et al, 2013).

Specific comment 6: I am puzzled by the large excess of AIF over PTEN that is required to induce comparably small effects on PTEN oxidation and PTEN enzymatic activity as shown in
Figure 3D,E. Is PTEN such a poor substrate or is it that the AIF effects on PTEN are non-enzymatic? Can the authors comment on this?

**RESPONSE:** In our previous Figure 3D, equal amount (100 ng) of AIF to PTEN could decrease ox-PTEN, which became significant with 300 ng of AIF (+++). According to the concern of this reviewer we repeated the experiment and showed that AIF decreased a biotinylated band matched with PTEN with a dose-independence, while PTEN-C124S mutant presented few oxidation (the current Fig 3D). The fact combined with the data that the NADH oxidase domain-deleted AIF mutant, AIFΔNADH, failed to generate the reduced form of PTEN (current Fig S2C), and like full-length AIF, AIFsh2 and AIFsh3 but not AIFsh could rescue AIF knockdown-induced PTEN oxidative modification and Akt activation (current Fig 3H), should support that the AIF effects on PTEN are enzymatic.

**Specific comment 7:** Based on the results of the current study one would assume that tumor cells gain a growth advantage by loss of AIF. Hence, AIF should frequently be downregulated or mutated in tumor cells. Yet, previous work (not cited by the authors: Jeong et al 2006, APMIS 144, 867) showed that about 80% of colorectal tumors have elevated AIF expression. Also in the Chinese colorectal cancer patient cohort studied by Shen and colleagues the majority of cases has high AIF expression and differences in overall survival depending upon the AIF status barely reach statistical significance (Figure 7M). AIF is a multifunctional protein and perhaps its pro-survival function is of greater importance in tumorigenesis than its role as antagonist of PI3K signaling. Thus, it could be informative to stratify patient cohorts according to the presence of PI3K mutations and then see how AIF expression relates to prognosis. The authors need to cite earlier work and address these issues in their discussion.

**RESPONSE:** Thanks for this good comment. We have pointed out that two previous reports showed that there was higher AIF expression in the majority of gastric carcinoma (Lee et al, 2006) and colorectal carcinoma (Jeong et al, 2006). The similar phenomena could also been found in our colorectal carcinoma cohort, although AIF expression was shown to be decreased in many other tumor types, contributing to their chemoresistance (Chiang et al, 2014; Sevioukova, 2011). To address the question that AIF is a multifunctional protein and perhaps its pro-survival function is of greater importance in tumorigenesis than its
role as antagonist of PI3K signaling, we mentioned that our unpublished data showed that hypoxia inhibited AIF expression, and low AIF expression existed in hypoxic regions of cancer tissues, although most cancer tissues expressed higher AIF. As a multifunctional protein, we proposed that its pro-survival function in cancer cells with high AIF expression contribute to tumorigenesis, and its role as antagonist of PI3K signaling in cancer cells with low AIF expression during hypoxic environment is correlated with EMT and metastasis of cancers. The related discussion was shown in the lines 7-15 of page 23.

Specific comment 8: In previous studies Prdx1 and Txnip were implicated in control of PTEN activity by reversible oxidation. Do these factors act on PTEN independently of AIF or is there a connection? The authors should address the issue of multiple independent PTEN control mechanisms or their potential interconnection in the Discussion.

RESPONSE: In the current discussion section, we addressed the comment as follows in the lines 9-17 of page 21: A previous report found that the peroxidase Prdx1, which catalyze peroxide reduction to balance cellular H\textsubscript{2}O\textsubscript{2} levels(Liu et al, 2012), interacts with and protects PTEN from oxidation-induced inactivation(Cao et al, 2009). Txnip was also reported to be required to maintain sufficient thioredoxin NADPH activity to reductively reactivate oxidized PTEN and oppose Akt downstream signaling in nonlipogenic tissues (Hui et al, 2008). It deserves to be further explored whether AIF, Prdx1 and/or Txnip are either independent or combined with each other to be implicated in control of PTEN activity by reversible oxidation.

Specific comment 9: The authors try to support their hypothesis that AIF counteracts EMT by GSEA using TCGA transcriptome data. Recent reports show that colorectal tumor preparations can be contaminated by stromal cells and that "EMT" signature gene expression in fact is due to the stromal contaminants (Kim and Verhaak, 2015 Nat Genetics, 47, 307). Caution is warranted when using bulk tumor transcriptome data to support gene expression changes in epithelium-derived tumor cells specifically. Immunohistochemical analyses of AIF and EMT marker coexpression in colorectal tumors could be an alternative.
RESPONSE: Thanks for the good suggestion. In the revised version (see line 18-23 of page 15), we stated that “Considering that colorectal tumor preparations can be contaminated by stromal cells and that EMT signature gene expression was due to the stromal contaminants (Kim & Verhaak, 2015)”. Thus, we also examined the correlation between AIF and E-cadherin by immunohistochemical analysis (IHC) in a group of colon cancer tissues, and confirmed that the expression of AIF is indeed positively correlated with that of E-cadherin (current Fig S4C-E), which supports the existence of these correlations in cancer cells rather than due to stromal contaminants.

Specific comment 10: In Figure 6A changes in ZEB1, VIM and FN1 expression are negligible on RNA level, but clearly seen in Western blot analyses. Furthermore, the authors claim that ZEB1 upregulation is a consequence of WNT/β-catenin pathway activation upon knockdown of AIF and suggest that ZEB1 expression is responsible for the induction of EMT. However ZEB1 is not an exclusive target of WNT signaling and ZEB1 is not the only inducer of EMT. Therefore, the authors should also examine the expression of SNAIL1, SLUG and TWIST1 and especially of miR200 family members. Downregulation of the latter could readily explain increased ZEB1 protein levels. Importantly, since the authors claim that AIF governs EMT and metastasis of cancer cells through WNT/β-catenin pathway activation they need to demonstrate that further upregulation of the already high levels of ZEB1 and the further promotion of EMT in their system indeed depends upon β-catenin/TCF activity. Otherwise, the relevant passages in the manuscript need to be rephrased to account for the fact that the authors’ claims are merely based on correlations.

RESPONSE: By the good comment, we also detected the expressions of other three main EMT activators including Twist, Snail and Slug besides ZEB1 upon AIF knockdown in SW620 cells. Our results showed that among these activators, only the expression of ZEB1 was elevated (Fig 6A/B and Fig S4F). We also pointed out that “it appeared that the increase of ZEB1 protein was more significant than its mRNA upon AIF knockdown (Fig 6A/B)” in lines 3-4 of page 16 of the revised version. In the future, we’ll answer whether this involves the miR200 family members. In spite of this, to address whether AIF knockdown-triggered EMT is a result of β-catenin signaling activation, we infected
two pairs of shRNA against β-catenin in AIF knockdown cells. Both shRNA almost completely reversed the increased ZEB1 protein and EMT program upon AIF silencing (current Figure 6F/G). Also, considering the comment, we did not emphasize the role of ZEB1 in AIF knockdown-induced EMT. The corresponding description could be seen in lines 4-13 of page 16.

Specific comment 11: Likewise, none of the genes examined in Figures 4 and 5 are under sole control of WNT signaling not even AXIN2 (see for example Morris et al 2008, Nature 455, 552; Hughes and Brady, 2005, Exp Cell Res 303, 32). To substantiate their claims the authors need to provide clear proof that the observed alterations in gene expression are due to changes in β-catenin/TCF activity.

RESPONSE: Considering the comment that none of these genes are under sole control of WNT/β-catenin signaling (Hughes & Brady, 2005; Morris et al, 2008), we knocked down β-catenin with shβ-catenin#2 in AIF silenced cells, and found that β-catenin knockdown almost completely blocked AIF silencing induced expression of all six genes (current Fig S3D). These data should support our claim for the role of activated β-catenin in the AIF knockdown-mediated expressions. The related data were described in line 23 of page 13 to line 4 of page 14 of this revised version.

Specific comment 12: The authors implicate β-catenin signaling in the phenotypic consequences of AIF knockdown because of increased AKT activity and the ensuing inhibitory phosphorylation of GSK3. Although this is a widely held belief, the architecture of the WNT/β-catenin pathway actually prohibits such crosstalk (Ng et al, 2009 J Biol Chem. 284, 35308 "Phosphatidylinositol 3-kinase signaling does not activate the WNT cascade"). In fact, there could be alternative mechanisms to activate β-catenin downstream of PTEN and the observed effects of AIF knockdown could be completely independent of β-catenin signaling (see for example Liliental et al, 2000, Curr Biol 10, 401; Myant et al 2013, Cell Stem Cell 12, 761). Therefore, to be able to maintain their mechanistic explanations the authors need to firmly establish β-catenin-dependency of the observed phenotypic changes in response to AIF depletion.

RESPONSE: Thanks for these suggestions. In this version, we pointed out that “Previously, it was also proposed that crosstalk between the PI3K and WNT pathways might be prohibited
and WNT-mediated transcriptional activity was not modulated by activation of the PI3K/Akt pathway (Ng et al, 2009)” (see lines 9-11 of page 22). But we think that our data from LY294002, an inhibitor of the PI3K/Akt pathway (Fig 5C), NAC treatment (Fig 5D) and overexpression of the PTEN-C71S mutant (Fig 5E), which reversed AIF knockdown-induced expressions of WNT signaling target genes, clearly support that AIF regulates β-catenin signaling through PTEN-PI3K/Akt pathway, as we discussed in the lines 12-15 of page 22. On the other hand, our new data from β-catenin knockdown assay (current Fig S3D, Figure 6F/G) should support β-catenin-dependency of the observed phenotypic changes in response to AIF depletion, as we responded to the last two specific comments of this reviewer.

Specific comment 13: In Figures 4 and 5 the authors analyze the expression of a set of six presumptive WNT/β-catenin target genes. However, sometimes one or more of the genes are omitted from the analyses (AXIN2, LEF1, MMP7; Figures 4G, 5A, 5C). I suggest to consistently show the data for all six genes. Moreover, since some of the expression changes observed are very small on RNA level (MMP7, AXIN2, ID2 in Figures 4E-G and 5D-F), irrespective of their statistical significance, I recommend to confirm the expression changes by Western blotting.

RESPONSE: Thanks. We have consistently showed the data for all six genes in the revised version. Because of the availability of antibodies, we only showed the AXIN2 protein expression in the current Fig S3B.

Specific comment 14: In many instances, the authors nicely substantiate their work by rescue experiments with different AIF constructs or oxidation-resistant PTEN. However, they deviate from this practice in Figure 7. To further support the relevance of the newly discovered AIF-PTEN axis in EMT and metastasis, at least the impact of combined AIF depletion and PTEN-C71S expression in the migration/invasion studies and, importantly, in xenotransplants should be shown. Can PTEN-C71S revert the cellular phenotypes of AIF loss-of-function? An informative extension of this would be to deplete both AIF and ZEB1 in these assays to demonstrate that ZEB1 is the critical factor downstream of AIF and PTEN.
**RESPONSE:** In the revised version, we also investigated the impact of combined AIF depletion and PTEN-C71S expression in xenografts, and the results showed that PTEN-C71S reversed the enhanced metastasis caused by AIF knockdown (current Fig 7H/I).

According to your specific comment 10, we did not emphasize the role of ZEB1 in AIF knockdown-induced EMT in the revised version. So we did not further study the role of ZEB1 in this time.

**Specific comment 15:** In Figure 7I, HT29 cells are used as a second model to study the impact of AIF knockdown on tumor formation and metastasis but only efficacy of the AIF knockdown is shown. Does reduction of AIF levels have the same functional consequences on PTEN activity, phosphorylation of AKT and GSK, and the expression of WNT target genes or EMT markers as in SW620?

**RESPONSE:** In the revised version, we performed experiments in HT29 cells on functional consequences of AIF knockdown on PTEN activity (current Fig 3K and Fig S2E), phosphorylation of Akt and GSK-3β (current Fig S2E), and the expression of WNT/β-catenin target genes (current Fig S3A) or EMT markers (current Fig S4A). All data showed the similar results as seen in SW620 cells.

**Specific comment 16:** The y-axes in Figure 7H and L are labeled identically but it is not clear whether the same thing is shown by the diamonds in both graphs, the number of mice with liver metastases or the number of metastatic nodules per liver. Furthermore, in Figure 7O it should be specified whether lymph node or distant organ metastasis was considered because the two have different prognostic values.

**RESPONSE:** Both Figure 7H and L (current Fig 7G and M) shows the number of metastatic nodules per liver. Figure 7O (current Figure 7P) shows the distant organ metastasis. The y-axes of these figures were more clearly labeled in the revised version.

**Specific comment 17:** Most of the figures are overcrowded and the lettering and lines are almost too faint. I am afraid that upon the necessary size reduction the figures will no longer be legible. The figures should be given a thorough work over to improve their quality and to eliminate some apparent errors. For example, in contrast to the statement in the legend, Figure 2E does not show SW620 cells but mitochondria. In Figures 2C, 2F, 5E and 5F positions of the side labels shifted, “Non-reducing” and “Reducing” were mixed
up, and the y-axis labels are incorrectly positioned, respectively. In Figure 5A the PTEN low and high status can only be guessed. Abbreviations in Figure 1J need to be explained in the legend. For the GSEA in Figure 4, panels H and I normalized enrichment scores (NES) and p-Values need to be shown (just as was done for Figure 6E). In both cases (Figures 4H,I and 6E) it should be indicated what is represented by the red/blue scale bars.

RESPONSE: Actually, we prepared all figures according to the requirement of the journal. Indeed, there are too many data in a figure. In our mind, the figures we uploaded were clear. We guess that PDF building damaged the quality. Therefore, we hope that the reviewer can see the original figures. We appreciate the reviewer to carefully check all these figures, and we have corrected the related concerns. In addition, we have deleted the previous Figure 5A.

Specific comment 18: Similarly, Figures S1 and S2 are very small and hardly legible when printed in their original size. Figure size and lettering need to be increased.

RESPONSE: Thanks! We have done my best to improve them.

Specific comment 19: I think there is a certain degree of redundancy provided by Figure 3 panels F/G, and I/L. The consequences of AIF knockdown and rescue could easily be put together without loss of clarity.

RESPONSE: Thanks. The previous Fig 3F/I has been put together as the current Figure 3F, and the experiments of the previous Fig 3G/L were re-performed and put together as the current Figure 3H.

Specific comment 20: Likewise, in Figure 4, the number of panels could be reduced by combining panels D with G (AIF knockdown and rescue) and panels E with F (overexpression of various AIF constructs).

RESPONSE: Thanks for the advice. In the revised version, we deleted the previous Figure 4F, and put the previous Fig 4G into supplemental Figures (current Fig S3D) to make Figure 4 more concise.

Specific comment 21: In Figure 7F serial sections should be shown and the tumor area be marked, especially in the E-cadherin-negative case.

RESPONSE: Ok, we have done it in current Fig 7E.
Specific comment 22: What is the reason for showing Figure 7G? It seems to me that it has no information value.

RESPONSE: We agree. So, we have deleted the previous Figure 7G in the revised version.

Specific comment 23: SW480 cells are mentioned in the Materials and Methods section but they are not used in the study.

RESPONSE: To detect the expression of AIF isoforms in the previous Figure 1L (current Figure S1B), SW480 cells were used. Therefore, we still mentioned it in the Materials and Methods section.

Specific comment 24: The authors do not provide a description of their construct targeting PTEN to mitochondria.

RESPONSE: Thanks! We provided a description of the construction of mito-PTEN in the revised Materials and Methods section (line 22-23 of page 24).

Specific comment 25: Several parts of the Materials and Methods sections are identical between the main manuscript and the Expanded View Content. These are unnecessary duplications and increase main manuscript length.

RESPONSE: Thank you for good suggestion. According to the editor’s suggestion, all materials and methods have been included in the main manuscript file of the revised version.

Specific comment 26: Figure S1H: Knockdown of AIF appears to have different effects on AKT and GSK3 phosphorylation (increase, decrease and neutral) in different cells which cannot simply be explained by the PTEN status. The authors should quantify changes in pAKT and pGSK3β in DU145, PC-3 and LNCaP cells and comment on the possibility that additional factors impinge on the functional connections between AIF, PTEN, AKT and GSK3.

RESPONSE: We have quantified the changes in pAkt and pGSK-3β in DU145, PC-3 and LNCaP cells in the revised version as shown in the current Fig S2F/G, and also described “Notably, AIF knockdown appeared to reduce Akt-S473 and GSK-3β phosphorylations in the PC3 cells, suggesting that additional factors impinge on the functional connections between AIF, Akt and GSK-3β in the PTEN-deficient cells.” in the lines 5-9 of page 11.
**Specific comment 27:** Table S2 does not provide sufficient data to allow evaluating the significance of the LC-MS/MS results. Furthermore, the initial interactor screen and the LC-MS/MS related methodology are not described in the manuscript. On the other hand, Table S2 also does not provide information that would be essential for the understanding of the study. Therefore, I suggest to either omit Table S2 or to provide additional information to make its inclusion worthwhile, including a detailed description of the experimental procedures. For example, what was the number of peptides found, the sequence coverage and significance scores, etc.? Moreover, the candidates could be sorted based on their intracellular localization.

**RESPONSE:** We have described the initial interactor screen and the LC-MS/MS related methodology in detail in line 3 of page 26 to line 20 of page 27, and according to the comment, we deleted the previous Table S2, and stated “data not shown” in the revised version.

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. As you will see, referee 1 still has a few suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. Referee 1 is asking for clarifications and discussions and also for more experimentation. However, referee 3 upon commenting on referee 1’s remaining concerns does not think that further experimentation is required. While the suggested experiments are certainly very welcome, they are not strictly necessary. But please discuss the remaining issues very clearly in the manuscript text.

I noticed that SF2 and SF5 do not fit on one page. Every figure must fit on a single page. Please either re-arrange the panels or split the figures into two. S5B does not mention "n" and does not specify the error bars, please add. EMBO press papers now integrate supplementary figures inline in the manuscript text such that they expand when clicked. Can you therefore please label your SF figures expanded view EV1, 2, etc figures both in the manuscript text and figure legends, upload a single file per figure and add the legends for EV figures to the end of your main manuscript file? We can currently only offer EV figures for 5 figure per manuscript. In case you will have more, please include the less relevant ones in the Appendix file. For more information please see our guide to authors at http://embor.embopress.org/authorguide#expandedview

I look forward to seeing a new and final version of your manuscript as soon as possible. Don't hesitate to contact me if you have any questions.

**REFEREE REPORTS:**

Referee #1:

This manuscript has greatly improved and the authors put clearly a significant effort in revising the manuscript.
In the reviewer's opinion however, there are still two issues, which need attention and need to be addressed.

1) The authors show that under oxidative stress the complex between AIF and PTEN seems unperturbed. How come then that under oxidative stress PTEN oxidation steadily increases despite AIF binding (S2I and 2J)? Is AIF function lost under oxidative stress? If that is true, a specific AIF oxidoreductase mutant (catalytically inactive) mutant needs to be used to address this issue.

That is also important because the authors mention in their response letter on several occasions that cancer cells presumably contain more ROS and therefore loss of AIF leads to PTEN oxidation. However, under basal conditions the levels of PTEN oxidation in the absence of AIF is very low (and correlates with low Akt phosphorylation). To clarify, cancer cells are sensitive to ROS (exogenous and endogenous) and produce endogenous ROS, therefore treatment of cancer cells with exogenous ROS is an important addition to the results.

2) The data shown in S2A and S2B use recombinant protein. In this experiment the PTEN band that migrates faster due to oxidation (as detected in cell lysates) is missing and the authors point to higher molecular bands as PTEN ox products. In addition, how is it explained that ox-PTEN forms in the non-reducing samples vanish when H2O2 is added? That is confusing.

In addition, since the blot in S2B is rather dark, it is difficult to assess if any PTEN AIF covalent structures exist.

This non-reducing membrane needs also be exposed to an anti-AIF antibody to address the question of covalent interaction. It also needs to be shown in its full size (below 60kDa).

Referee #2:

The authors have addressed appropriately the issues raised in the review

Referee #3:

Comments on manuscript number: EMBOR-2015-40536V2 "AIF Governs Tumor Metastasis by Protecting PTEN from Oxidation" by Shao-Ming Shen and co-authors

I have carefully read the revised version of the manuscript and the authors' response to my comments about the original version of their work. With the exception of only a few minor issues that would have only little impact on the key findings of the revised manuscript, the authors successfully and rather satisfyingly addressed all my previous concerns. Importantly, a significant number of additional experiments with the HT29 colorectal cancer cell line was added throughout the entire manuscript which definitely broadens the significance and impact of the study. Likewise, the mechanistic explanations for the observed phenotypic changes upon AIF knockdown were corroborated by adding shRNA experiments for β-catenin to prove the causative involvement of β-catenin-mediated signaling. Although it is still unclear whether the functional interaction between PTEN and AIF occurs in mitochondria and/or elsewhere, to solve this issue would not make a difference with respect to the main message of the manuscript. At this point, if anything, the manuscript could only be improved by some language editing and a spelling check. Nonetheless, the manuscript has greatly improved and I recommend its publication in EMBO reports.

Comments by Referee #3 on Referee #1’s concerns:
1) The authors show that under oxidative stress the complex between AIF and PTEN seems unperturbed. How come then that under oxidative stress PTEN steadily increases in the presence of AIF (S2I and 2J)? Is AIF function lost under oxidative stress? Is the oxidoreductase activity of AIF required to protect PTEN from oxidation? As stated in the first review, a AIF catalytically inactive mutant can address this issue.

Reviewer #1 touches a point which has puzzled me already during the first round of reviewing, namely that from the point of view of an enzymatic reaction AIF has an apparently rather low efficiency or low capacity to protect PTEN against oxidation. I think this is the explanation for the results shown in Figure S2IJ which indicate that the AIF protective system appears to be easily exhausted in the presence of exogenous H2O2. To address the issue whether AIF enzymatic activity is required, the authors use the rescue experiment shown in Figure 3H where AIFsh lacking the oxidoreductase domain fails to protect PTEN against oxidation. An inactivating point mutation in the catalytic center would be more elegant and convincing but to me AIFsh fulfills its purpose. To sum it up, in my opinion no additional experiments would be necessary.

2) The data shown in S2A and S2B use recombinant protein. Where is the PTEN faster migrating band usually detected in cell lysates? Also, how come that ox-PTEN forms in the non-reducing samples vanish when H2O2 is added? That is confusing. Since the blot in S2B is rather dark, it is difficult to assess if any PTEN AIF covalent structures exist.

In my opinion the authors accurately describe the experiment and their observations including the disappearance of a band around 70 kDa on page 9 of their manuscript starting from line 14. To me, it is conceivable that recombinant PTEN exhibits a pattern of oxidation that differs from what can be observed in mammalian cells. A potential explanation could be aggregation of oxidized forms that is exacerbated in the presence of H2O2 to an extent where PTEN-ox no longer enters the gel. The important aspect is that the addition of AIF can at least partially restore reduced PTEN. I think further experimentation is not necessary but nonetheless, the authors could point out the differences in migration between recombinant and cellular PTEN and provide potential explanations for this (including the sink of ox-PTEN in the presence of H2O2) in the text.

2nd Revision - authors' response

Detailed response to referees’ comments

Referee #1:
This manuscript has greatly improved and the authors put clearly a significant effort in revising the manuscript. In the reviewer's opinion however, there are still two issues, which need attention and need to be addressed.

RESPONSE: Thanks for the reviewer's evaluation.

concern 1) The authors show that under oxidative stress the complex between AIF and PTEN seems unperturbed. How come then that under oxidative stress PTEN oxidation steadily increases despite AIF binding (S2I and 2J)? Is AIF function lost under oxidative stress? If that is true, a specific AIF oxidoreductase mutant (catalytically inactive) mutant needs to be used to address this issue. That is also important because the authors mention in their response letter on several occasions that cancer cells presumably contain more ROS and therefore loss of AIF leads to PTEN oxidation. However, under basal conditions the levels of PTEN
oxidation in the absence of AIF is very low (and correlates with low Akt phosphorylation). To clarify, cancer cells are sensitive to ROS (exogenous and endogenous) and produce endogenous ROS, therefore treatment of cancer cells with exogenous ROS is an important addition to the results.

**RESPONSE:** We completely agree with the explanation on the concern from referee 3, as shown in the following statement of referee 3.

**Concern 2)** The data shown in S2A and S2B use recombinant protein. In this experiment the PTEN band that migrates faster due to oxidation (as detected in cell lysates) is missing and the authors point to higher molecular bands as PTEN ox products. In addition, how is it explained that ox-PTEN forms in the non-reducing samples vanish when H2O2 is added? That is confusing. In addition, since the blot in S2B is rather dark, it is difficult to assess if any PTEN AIF covalent structures exist. This non-reducing membrane needs also be exposed to an anti-AIF antibody to address the question of covalent interaction. It also needs to be shown in its full size (below 60kDa).

**RESPONSE:** We also completely agree with the explanation on the concern from referee 3, as shown in the following statement of referee 3. In the revised version, we have revised the related words to clearly described our view in bottom 12 lines of page 9: Consistent with the previous report[8], bacterially expressed PTEN was oxidized to different extent including a band around 70 kDa during the in vitro purification so that the reduced form of PTEN (~60 kDa) was barely detectable under non-reducing condition (Fig EV2A and EV2B). Upon exposure to exogenous H2O2, these in vitro purified PTEN appeared to be highly oxidized, with the disappearance of the oxidized band around 70 kDa under non-reducing condition (Fig EV2B), for which a potential explanation could be aggregation of oxidized forms that is exacerbated in the presence of H2O2 to an extent where the oxidized PTEN was difficult or no longer enter the gel. However, dithiothreitol (DTT) treatment resulted in the appearance of a single band of ~60 kDa PTEN (Fig EV2A). The fact that the putative oxidoreductase domain of AIF interacts with the phosphatase domain of PTEN promoted us to ask whether AIF regulates the redox state of PTEN. Indeed, when the in vitro purified PTEN was incubated with the recombinant AIF under
non-reducing condition, a band matched with the reduced form of PTEN could be clearly seen (Fig 3C, Fig EV2B and Fig EV2C).

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I have carefully read the revised version of the manuscript and the authors' response to my comments about the original version of their work. With the exception of only a few minor issues that would have only little impact on the key findings of the revised manuscript, the authors successfully and rather satisfyingly addressed all my previous concerns. Importantly, a significant number of additional experiments with the HT29 colorectal cancer cell line was added throughout the entire manuscript which definitely broadens the significance and impact of the study. Likewise, the mechanistic explanations for the observed phenotypic changes upon AIF knockdown were corroborated by adding shRNA experiments for β-catenin to prove the causative involvement of β-catenin-mediated signaling. Although it is still unclear whether the functional interaction between PTEN and AIF occurs in mitochondria and/or elsewhere, to solve this issue would not make a difference with respect to the main message of the manuscript. At this point, if anything, the manuscript could only be improved by some language editing and a spelling check. Nonetheless, the manuscript has greatly improved and I recommend its publication in EMBO reports.

RESPONSE: We appreciate the referee’s serious and professional comments. In the revised version, we have also done our best to improve language editing and spelling. In addition, we also pointed out that “although the functional interaction between PTEN and AIF in mitochondria and/or elsewhere needs to further addressed” on the last paragraph of discussion section.

Comments by Referee #3 on Referee #1’s concerns:
1) The authors show that under oxidative stress the complex between AIF and PTEN seems unperturbed. How come then that under oxidative stress PTEN steadily increases in the presence of AIF (S2I and 2J)? Is AIF function lost under oxidative stress? is the oxidoreductase activity of AIF required to protect PTEN from oxidation? As stated in the first review, a AIF catalytically inactive mutant can address this issue.
Reviewer #1 touches a point which has puzzled me already during the first round of reviewing, namely that from the point of view of an enzymatic reaction AIF has an apparently rather low efficiency or low capacity to protect PTEN against oxidation. I think this is the explanation for the results shown in Figure S2I,J which indicate that the AIF protective system appears to be easily exhausted in the presence of exogenous H2O2. To address the issue whether AIF enzymatic activity is required, the authors use the rescue experiment shown in Figure 3H where AIFsh lacking the oxidoreductasse domain fails to protect PTEN against oxidation. An inactivating point mutation in the catalytic center would be more elegant and convincing but to me AIFsh fulfills its purpose. To sum it up, in my opinion no additional experiments would be necessary.

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In my opinion the authors accurately describe the experiment and their observations including the disappearance of a band around 70 kDa on page 9 of their manuscript starting from line 14. To me, it is conceivable that recombinant PTEN exhibits a pattern of oxidation that differs from what can be observed in mammalian cells. A potential explanation could be aggregation of oxidized forms that is exacerbated in the presence of H2O2 to an extent where PTEN-ox no longer enters the gel. The important aspect is that the addition of AIF can at least partially restore reduced PTEN. I think further experimentation is not necessary but nonetheless, the authors could point out the differences in migration between recombinant and cellular PTEN and provide potential explanations for this (including the sink of ox-PTEN in the presence of H2O2) in the text.

**RESPONSE:** We completely agree with your response. Thanks a lot.

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3rd Editorial Decision

26 August 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.