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FATE1 antagonizes calcium- and drug-induced apoptosis by uncoupling ER and mitochondria

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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.)

1st Editorial Decision 08 November 2015

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the three reports from the referees that were asked to assess it.

As the detailed reports are pasted below I would prefer not to repeat them here, but you will see that the reviewers, in principle, agree on the potential interest of the findings. However, they also feel that as it stands, the data on a potential role for FATE1 in regulating apoptosis by uncoupling ER and mitochondria is not conclusive yet.

The main issue raised by referees 1 and 3 is that the study relies too heavily on the use of protein overexpression and that most experiments would need to be repeated at the level of endogenous proteins. Without those data, the physiological relevance of the observations remains unclear and the reviewers do in this case not support publication in EMBO reports. In addition, the referees also raise a number of other technical and experimental issues, which are clearly outlined in their reports.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript may become suitable for publication in EMBO reports. However, given the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referee concerns must be addressed in full and their suggestions (as detailed above and in the referees' reports) taken on board.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly, but in essence, manuscripts need to be accepted within six months after the initial invitation to revise the study; if they are submitted/accepted later than that, their novelty will need to be assessed at the time of resubmission.

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

REFEREE REPORTS

Referee #1:

In this study, Doghman M et al addresses the role of FATE1, a cancer testis antigen, in the coupling of mitochondria/ER contact sites and its potential role in calcium induced apoptosis. FATE1 expression is primarily limited to adrenal and gonadal cells, regulated by the transcription factor SF-1. In this study, the authors claim that FATE1 is an OMM protein, anchored by its hydrophobic C-terminus domain. Expression of FATE1 leads to a decrease of the mito/ER contact sites, accompanied by a diminution of the mitochondrial calcium uptake leading to an apoptosis H202-dependent decreased. Although the subject matter is intriguing, there are number of technical concerns with the study, leading to inconsistencies in the data. The functional role of FATE1 in the modulation of ER/mito contacts is not well established, limiting my enthusiasm for the study.

Specific comments:
1. The expression of tagged FATE1 in HeLa cells reveals convincing mitochondrial targeting in Figure 1, with immunogold staining and confocal microscopy. However, the fractionation of endogenous FATE-1 induced in ACC H295R/TR SF-1 cells revealed absolutely no localization to the crude or purified mitochondrial compartment, rather the protein was highly enriched in the ER fraction, along with a number of established ER markers. The authors suggest a function for the small amount of FATE1 found in the MAM fraction, however this data does not convince me that FATE1 is enriched, or functionally relevant in that fraction. To me, the data question the results obtained with the tagged form, since such a clear mitochondrial localization by IF and EM does not reflect the fractionation of the endogenous protein. This is of serious concern. (In general the authors must label all of their images in a way that clearly indicates whether they are staining tagged FATE1 or the endogenous.)

2. The authors attempted to map the tail-anchor signal of FATE1 in HeLa cells expressing GFP tagged forms of FATE1. Essentially all of the images presented are showing highly overexpressed cells, and it is difficult to conclude much from these studies. I appreciate that tail anchors can often be dual targeted to the ER and mitochondria (Bcl-2 is a good example), but this figure is not easily interpretable. Higher magnifications and lower expression levels are required, complemented with biochemical fractionations. In addition, since FATE1 is expressed only in adrenal and gonadal cells, the HeLa cell localization may not reflect it's true functional localization. The targeting experiments should be performed in a relevant cell type, like their ACC line H295R.

3. The authors looked at the potential role of FATE1 at the MAM based on the interacting proteins identified in a co-IP experiment from cells expressing tagged FATE1. None of these interactions were further validated, or shown to be functionally required for any of the later phenotypes they investigate. The levels of overexpression are also of concern, as chaperone-related proteins could certainly bind to excess membrane inserted proteins.

4. The authors should carefully examine whether overexpression or silencing of FATE1 alters the mitochondrial morphology. To my eyes, many of the cells shown may have a clumping and/or fragmented phenotype, but again, the very low magnification and quality of the images makes this

difficult to discern. If FATE1 is related to Mff, there remains the possibility for a role in morphology, at the ER and/or the mitochondria.

5. In performing the mitochondrial calcium uptake experiments the authors note a decrease in mitochondrial calcium within the FATE1 expressing cells (and increase in siFATE1), with no change in the cytosolic calcium levels. However, the important control here is the total ER calcium content. Should FATE1 expression alter the function of the ER, it is very likely that the calcium stores will be reduced, which could explain the reduction in mitochondrial calcium uptake. The quantification of the Manders co-efficient shown in Fig 3B is not convincing at the magnification and resolution shown here. This data should be confirmed using EM approaches. The split luciferase experiment shows a single cell, and I have no idea how robust that observation may be.

6. As a point of interest, if FATE1 expression is limited to steroidogenic cell types, calcium may not be the relevant metabolite that would flux between the ER and mitochondria. Instead wouldn't it make sense to look at steroidogenesis itself - where ER derived cholesterol must get into the mitochondria through the StAR pathway?

7. The authors did not provide any validation for the expression of FATE1 in the H295R/TR N-FLAG FATE1 dox inducible system. Similarly, the efficiency of the silencing must be demonstrated in each experiment.

8. Particularly in Figure 4, the authors must include FATE1 and SF1 blots to confirm the induction of expression.

9. In Figure 5 the authors measure caspase activity, which is modestly changed, but this must be expanded to confirm whether there is actually any meaningful change in cell death. It is also unclear why they chose to examine death triggers that are not particularly reliant on calcium flux, as would triggers like ceramide or thapsigargan. These minor changes in activity may really reflect alterations in ER or mitochondrial function, rather than reflecting any role for FATE1 at ER/mito contact sites. Overall, this figure is not very convincing.

10. I appreciate that Figure 6 addresses the linkages of FATE1 to cancer progression, a point that has been highlighted in the literature. However, this does not help to convince me that the function of FATE1 is 1) at the mitochondria, 2) at ER contacts and/or 3) required for calcium uptake into mitochondria.

Referee #2:

The study by Doghman et al examines the mechanism by which FATE1 regulates apoptotic cell death in cancer cells. The authors reveal that FATE is localized at the MAMs of the OMM and modulates mitochondrial calcium uptake from the ER. The results show that increase expression of FATE1 decreases mito-ER contact and thus decreases mitochondrial calcium uptake, while knock-down of FATE1 enhances calcium uptake from the ER. The authors also show that increased FATE expression reduces apoptotic cell death. Finally, some interesting patient data showing an inverse correlation between FATE1 expression and survival are included which add further interest and significance to these findings.

Overall, this is a very nice nice paper addressing a novel question regarding the pro survival function of FATE1. The techniques used are all appropriate to address the questions being asked and the experiments are generally well done. There are 2 issues that the authors should address to strengthen the paper:

The cell death studies are conducted using mitotane, peroxide and STS and results for some experiments are not very robust (eg. Mitotane). Given that FATE1 regulates mitochondrial calcium uptake from the ER, the authors could do some more relevant experiments to directly test whether FATE1 protects cells against this mode of cell death. For example, if they could induce cell death by increasing expression of the BH3-only protein BIK (for example) which induces BAX-dependent death by increasing mitochondrial calcium uptake from the ER, these results would be more

meaningful.

The sample numbers in Fig 6b for non-steroidogenic tumors are too low and thus not very meaningful.

Referee #3:

In their manuscript "FATE1 antagonizes calcium- and drug-induced apoptosis by uncoupling ER and mitochondria" Doghman et al. describe their analysis on the cancer-testis antigen encoded by FATE1. Based on the performed experiments the authors conclude that the FATE1 protein resides in the outer mitochondrial membrane and localizes to mitochondria-associated-membranes (MAM) that represent close contacts between the ER and mitochondria. Furthermore, the authors conclude that varying FATE1 levels impact the distance between ER and mitochondria, which has consequences for mitochondrial Ca2+-import and the sensitivity to mitochondrial Ca2+-dependent proapoptotic stimuli.

ER-mitochondria interfaces have in recent years emerged as important cellular structures that have been implicated in mitochondrial fission and calcium and lipid transport between the ER and mitochondria. Given the importance of these processes for cellular function, the identification of the structural and regulatory components of ER-mitochondria interfaces is of outstanding cell biological interest. Therefore, the identification of FATE1 as a regulatory component of ER-mitochondria interfaces would be a very valuable and important contribution.

This manuscript is well written and the data are of good quality. The authors conclude based on their data that FATE1 is a component that regulates ER-mitochondria interfaces. This conclusion hinges on the co-immunoprecipitation experiments identifying ER-resident proteins as interacting proteins of FATE1, the subcellular fractionation study that identifies FATE1 as a component of the MAM fraction and the microscopic analyses on ER-mitochondria interfaces that suggest that these interfaces are modulated dependent on FATE1 levels. However at this point, the provided data do not sufficiently support the conclusions drawn.

1. It is interesting that MAM-enriched proteins are co-immunoprecipitated with FATE1; however, the data provided in the current manuscript make it impossible to evaluate the degree of interaction. It is difficult to assess, whether any controls have been performed (pull-down experiment in an untransfected cell-line) that show that the interactions are specific. Given the importance of a physical interaction of FATE1 and ER-resident proteins for the conclusion, reciprocal pull-down experiments with FATE1 and other MAM components would likewise be greatly supportive. Such experiments should be included in the main part of the manuscript.

2. The subcellular fractionation studies in Figure 3a are interpreted to suggest that FATE1 localizes into MAMs. It is, however, puzzling that in this experiment FATE1 is enriched in the ER fraction, compared to the 'crude mitochondrial' fraction. This is at odds with the microscopy data provided in Figure 1d, 1e and many parts of Figure 2 that show that the majority of FATE1 is at the OMM. Furthermore, Figure 3a shows that FATE1 is markedly dis-enriched from the purified mitochondrial fraction. The data presented in 3a suggests that the majority of FATE1 localizes to the ER and is enriched at ER-mitochondria interfaces. These results are difficult to reconcile with the microscopy data that show a broad distribution of FATE1 across the whole mitochondrial organelle and not a focal localization to ER-mitochondria contact sites. These discrepancies raise doubts about the subcellular fractionation experiments and the microscopy data and must be experimentally clarified. A triple labeling experiment examining mitochondria, ER and FATE1 localization might more strongly support the proposed enrichment of FATE1 at ER-mitochondria interfaces.

3. The data provided in Figure 3b are interpreted to suggest that ER-mitochondrial distance increases in response to FATE1 overexpression. Light fluorescence microscopy does not have the spatial resolution necessary to clearly distinguish between ER and mitochondria that are in the vicinity of one another and actual contact sites between both organelles. At this point it is entirely possible that subtle changes in organelle morphology, which could be a result of FATE overexpression, would result in the same difference in colocalization of mitochondria and ER determined by this method. The authors try to support these data by utilizing a split-GFP system, in

which complementing parts of GFP are targeted to the mitochondrial outer membrane and the ER. This is a very interesting assay; however, by following the provided reference, no evidence can be found that sufficiently validates this method, specifically for ER-mitochondria interfaces. Therefore, concluding altered ER-mitochondria distance based on this assay seems premature. Further support for altered ER-mitochondria contacts dependent on FATE1 levels must be provided. Such experiments could for example utilize microscopy techniques with higher spatial resolution.

In summary, while this manuscript attempts to assign FATE1 a direct role in mitochondrial-ER attachment site regulation, further experiments must be done to support this conclusion. Without such experiments the conclusion that FATE1 affects mitochondrial Ca2+-import and the sensitivity to mitochondrial Ca2+-dependent proapoptotic stimuli by modulating ER-mitochondria contacts still remains too speculative and could as well be explained by FATE1 levels broadly affecting other aspects of mitochondrial biology.

Other comments:
• Enlarged regions in the microscopy data in Figure 1 should be provided to allow assessment of the localization of FATE1 relative to the other markers.
• In Figure 2, a panel needs to be added to schematically illustrate the mutants used in the study.
• In Figure 3b, enlarged regions need to be shown.
• Why is mt-CA2+ content lower in cells transfected with n-Flag FATE1? This needs to be explained and discussed.
• Figure 4 should be moved to the supplement. Also, a quantification of the detected signals might support the authors' claims, as small differences in the levels can be observed for some of the analyzed proteins. A figure for the co-immunoprecipitation data should be included in the main part of the manuscript.

1st Revision - authors' response 08 February 2016

We appreciate the positive feedback by all three reviewers and are grateful for the comments we received on the first version of our manuscript that helped us to considerably improve it. In the revised version of our manuscript we were able to address all criticisms raised by the reviewers, as detailed here below.

As a general point, we would like to underline here that most results shown in this paper were obtained in our H295R/TR SF-1 cell line model of adrenocortical carcinoma, where expression of endogenous FATE1 is regulated by increased doxycycline-dependent SF-1 transcription factor dosage, since, as we have shown before (refs. 6 and 7 in our manuscript), FATE1 expression is regulated by SF-1 in adrenocortical cancer cells. The data obtained were confirmed in the stable H295R/TR FATE1 and N-Flag-FATE1 cell lines we generated displaying doxycycline-dependent moderate FATE1 expression from an integrated transgene (which allowed us to study the effects of FATE1 independently from the effects of other SF-1 - regulated gene products) and, limited to some subcellular localization experiments for FATE1 mutants, in transiently transfected HeLa cells. In addition, the specificity of the results obtained was confirmed by selective FATE1 knockdown in the H295R/TR SF-1 cell line.

Here follows a detailed, point-by-point rebuttal to the reviewers' comments and criticisms:

Referee #1

In this study, Doghman M et al addresses the role of FATE1, a cancer testis antigen, in the coupling of mitochondria/ER contact sites and its potential role in calcium induced apoptosis. FATE1 expression is primarily limited to adrenal and gonadal cells, regulated by the transcription factor SF-1. In this study, the authors claim that FATE1 is an OMM protein, anchored by its hydrophobic C-terminus domain. Expression of FATE1 leads to a decrease of the mito/ER contact sites, accompanied by a diminution of the mitochondrial calcium uptake leading to an apoptosis H202-dependent decreased. Although the subject matter is intriguing, there are number of technical concerns with the study, leading to inconsistencies in the data. The functional role of FATE1 in the modulation of ER/mito contacts is not well established, limiting my enthusiasm for the study.
Specific comments:

1. The expression of tagged FATE1 in HeLa cells reveals convincing mitochondrial targeting in Figure 1, with immunogold staining and confocal microscopy. However, the fractionation of endogenous FATE-1 induced in ACC H295R/TR SF-1 cells revealed absolutely no localization to the crude or purified mitochondrial compartment, rather the protein was highly enriched in the ER fraction, along with a number of established ER markers. The authors suggest a function for the small amount of FATE1 found in the MAM fraction, however this data does not convince me that FATE1 is enriched, or functionally relevant in that fraction. To me, the data question the results obtained with the tagged form, since such a clear mitochondrial localization by IF and EM does not reflect the fractionation of the endogenous protein. This is of serious concern. (In general the authors must label all of their images in a way that clearly indicates whether they are staining tagged FATE1 or the endogenous)

We agree with this reviewer that the results of the subcellular fractionation method used for MAM preparation showing endogenous FATE1 enrichment in the ER and in MAM fractions (Fig. 2E) are apparently at odds with the *in situ* localization of the endogenous FATE1 protein at the level of the mitochondrial outer membrane extensively shown in Fig. 1, 2B, 3E, EV1 and EV2. However, it is important to underline that the same cell line (Dox-treated H295R/TR SF-1 cells) was used to assess FATE1 subcellular localization using both biochemical and morphological methods and that MAM preparation was performed using a well-established method (see ref. 48 in our paper) and repeated several times in our laboratory, always producing the same results, as shown in Fig. 2E. Furthermore, control proteins known to be enriched in the MAM fraction (SERCA2, S1R, VDAC) and a mitochondrial OMM protein (TOM20) show the respective expected localization after our MAM preparation procedure. We interpret these apparently conflicting results in the sense that during the MAM purification procedure tight interactions of FATE1 with MAM and ER proteins (including EMD, which is highly enriched in MAM as shown in Fig. 2E) can "strip" it from the mitochondrial outer membrane, to which the protein is associated through a short C-terminal transmembrane domain. We have discussed our findings in the Discussion section of our manuscript at page 12.

2. The authors attempted to map the tail-anchor signal of FATE1 in HeLa cells expressing GFP tagged forms of FATE1. Essentially all of the images presented are showing highly overexpressed cells, and it is difficult to conclude much from these studies. I appreciate that tail anchors can often be dual targeted to the ER and mitochondria (Bcl-2 is a good example), but this figure is not easily interpretable. Higher magnifications and lower expression levels are required, complemented with biochemical fractionations. In addition, since FATE1 is expressed only in adrenal and gonadal cells, the HeLa cell localization may not reflect it's true functional localization. The targeting experiments should be performed in a relevant cell type, like their ACC line H295R.

In compliance with the suggestions of this reviewer, in the revised version of our manuscript we show the localization of EGFP-FATE1 fusion proteins in the H295R/TR SF-1 cell line (Fig. 2B and Fig. EV1). On the other hand, this localization is totally consistent with the localization of the same EGFP-fusion proteins in transiently transfected HeLa cells (Fig. EV2). Biochemical fractionation of HeLa cells transfected to express EGFP-FATE1 fusion proteins showed results consistent with fluorescence analysis (Fig. EV3). In particular, this method confirmed the loss of specific mitochondrial (heavy membrane) localization for the FATE1 L151D mutant.

3. The authors looked at the potential role of FATE1 at the MAM based on the interacting proteins identified in a co-IP experiment from cells expressing tagged FATE1. None of these interactions were further validated, or shown to be functionally required for any of the later phenotypes they investigate. The levels of overexpression are also of concern, as chaperone-related proteins could certainly bind to excess membrane inserted proteins.

In the revised version of our manuscript we show the results of a coimmunoprecipitation experiment performed in Dox-treated H295R/TR N-Flag FATE1 cells (stable Dox-inducible clone expressing moderate amounts of FATE1 after Dox treatment) where FATE1 is found to interact specifically with the ER and MAM-localized protein EMD, one of the FATE1-interacting partners we identified by mass spectrometry (Fig. 2C). In addition, FATE1 – EMD interaction is confirmed by data present

in databases from high-throughput yeast two-hybrid screening campaigns (ref. 17 in our manuscript).

4. The authors should carefully examine whether overexpression or silencing of FATE1 alters the mitochondrial morphology. To my eyes, many of the cells shown may have a clumping and/or fragmented phenotype, but again, the very low magnification and quality of the images makes this difficult to discern. If FATE1 is related to Mff, there remains the possibility for a role in morphology, at the ER and/or the mitochondria.

In the revised manuscript we have included data showing that FATE1 expression reduces mitochondrial fragmentation compared to basal conditions both in H295R/TR N-Flag FATE1 and H295R/TR SF-1 cells (Fig. 3E-G) without affecting mitochondrial membrane potential (Fig. 3H). The relationship between mitochondrial morphology and modulation of ER-mitochondria interactions is complex, but our data are in agreement with the finding that cell death and mitochondrial Ca^{2+} uptake, which are counteracted by FATE1 expression, are usually linked to an increase in mitochondrial fragmentation.

5. In performing the mitochondrial calcium uptake experiments the authors note a decrease in mitochondrial calcium within the FATE1 expressing cells (and increase in siFATE1), with no change in the cytosolic calcium levels. However, the important control here is the total ER calcium content. Should FATE1 expression alter the function of the ER, it is very likely that the calcium stores will be reduced, which could explain the reduction in mitochondrial calcium uptake. The quantification of the Manders co-efficient shown in Fig 3B is not convincing at the magnification and resolution shown here. This data should be confirmed using EM approaches. The split luciferase experiment shows a single cell, and I have no idea how robust that observation may be.

As shown in the Supplementary Information in the previous version of our manuscript, FATE1 expression does not alter ER calcium concentration, both in H295R/TR N-Flag FATE1 and H295R/TR SF-1 cells. These data are shown in Fig. EV4A and B of the revised manuscript. In addition, the effect of FATE1 expression on mitochondrial Ca*2+* uptake is lost after treatment of digitonin-permeabilized Dox-treated H295R/TR N-Flag FATE1 cells with cyclopiazonic acid, a specific inhibitor of sarco-endoplasmic reticulum Ca^{2}+-ATPases (Fig. EV4C, D). These data show that the effect on Ca^{2+} transfer into mitochondria triggered by FATE1 expression is MAM-dependent.
We would like to underline that the results about the effect of FATE1 expression on ER-mitochondria colocalization using the Manders' method in H295R/TR N-Flag FATE1 cells shown in Fig. 3A were confirmed by a split-GFP method whose results are shown in Fig. 3B. Importantly, those data do not involve the analysis of a single, but of 90 different cells, as shown in the histogram present in Fig. 3B. In addition, in compliance with the suggestion of this reviewer, in the revised version of our manuscript we have added the results of EM quantification of ER-mitochondria contacts in basal conditions and after Dox treatment both in H295R/TR N-Flag FATE1 and H295R/TR SF-1 cells. Both the number of ER-mitochondria contacts and the number of mitochondria displaying ER contact sites were taken into account (Fig. 3C, D). The EM data confirmed the results found using the two other approaches, that is that FATE1 expression decreases ER-mitochondria interaction.

6. As a point of interest, if FATE1 expression is limited to steroidogenic cell types, calcium may not be the relevant metabolite that would flux between the ER and mitochondria. Instead wouldn't it make sense to look at steroidogenesis itself - where ER derived cholesterol must get into the mitochondria through the StAR pathway?

We thank the reviewer for this interesting comment, which prompted us to measure aldosterone, cortisol and DHEAS production in basal conditions and after angiotensin II (AII) or forskolin (FSK) stimulation of H295R/TR SF-1 cells nucleofected with a control or a FATE1-specific siRNA and cultured with or without Dox. FATE1 knockdown increased the production of all three steroids in AII and FSK-stimulated cells. These data (shown in Fig. EV5) are again consistent with the inhibitory function of FATE1 on ER-mitochondria interactions since steroidogenic stimulation

through the Steroidogenic Acute Regulatory protein (StAR) requires interaction with MAM proteins, as shown by recent studies (refs. 23, 24 in our manuscript).

7. The authors did not provide any validation for the expression of FATE1 in the H295R/TR N-FLAG FATE1 dox inducible system. Similarly, the efficiency of the silencing must be demonstrated in each experiment.

We have shown Dox-regulated expression of SF-1 and FATE1 proteins in H295R/TR SF-1, H295R/TR FATE1 and H295R/TR N-Flag FATE1 cells in Fig. 1B and validated the efficiency of FATE1 knockdown in H295R/TR SF-1 cells (Fig. 1A).

8. Particularly in Figure 4, the authors must include FATE1 and SF1 blots to confirm the induction of expression.

As reported before, we have shown Dox-regulated expression of SF-1 and FATE1 proteins in H295R/TR SF-1, H295R/TR FATE1 and H295R/TR N-Flag FATE1 cells in Fig. 1B.

9. In Figure 5 the authors measure caspase activity, which is modestly changed, but this must be expanded to confirm whether there is actually any meaningful change in cell death. It is also unclear why they chose to examine death triggers that are not particularly reliant on calcium flux, as would triggers like ceramide or thapsigargan. These minor changes in activity may really reflect alterations in ER or mitochondrial function, rather than reflecting any role for FATE1 at ER/mito contact sites. Overall, this figure is not very convincing.

In compliance with the suggestion of this reviewer, in the revised manuscript we have included data showing that FATE1 expression in H295R/TR N-Flag FATE1 cells also decreases cell death (as measured by caspase 3/7 activity) induced by C2-ceramide (Fig. 4D) and data from flow cytometric TUNEL analysis showing a decrease in apoptosis induced by H_2O_2 and C2-ceramide, but not staurosporin, in the same cell line (Fig. 4E).

10. I appreciate that Figure 6 addresses the linkages of FATE1 to cancer progression, a point that has been highlighted in the literature. However, this does not help to convince me that the function of FATE1 is 1) at the mitochondria, 2) at ER contacts and/or 3) required for calcium uptake into mitochondria.

We hope that the wealth of new data added to the new version of our manuscript, which all converge to the same conclusions, will now help to convince this reviewer about the robustness of our findings.

Referee #2

The study by Doghman et al examines the mechanism by which FATE1 regulates apoptotic cell death in cancer cells. The authors reveal that FATE is localized at the MAMs of the OMM and modulates mitochondrial calcium uptake from the ER. The results show that increase expression of FATE1 decreases mito-ER contact and thus decreases mitochondrial calcium uptake, while knock-down of FATE1 enhances calcium uptake from the ER. The authors also show that increased FATE expression reduces apoptotic cell death. Finally, some interesting patient data showing an inverse correlation between FATE1 expression and survival are included which add further interest and significance to these findings.

Overall, this is a very nice paper addressing a novel question regarding the pro survival function of FATE1. The techniques used are all appropriate to address the questions being asked and the experiments are generally well done. There are 2 issues that the authors should address to strengthen the paper:

The cell death studies are conducted using mitotane, peroxide and STS and results for some experiments are not very robust (eg. Mitotane). Given that FATE1 regulates mitochondrial calcium uptake from the ER, the authors could do some more relevant experiments to directly test whether FATE1 protects cells against this mode of cell death. For example, if they could induce cell death by increasing expression of the BH3-only protein BIK (for example) which induces BAX-dependent death by increasing mitochondrial calcium uptake from the ER, these results would be more meaningful.

We thank this reviewer for his/her very positive appreciation of our study. As reported in the manuscript, we have investigated the effect of FATE1 expression on mitotane-induced ACC cell death (Fig. 4G, H) since this is the most widely used chemotherapeutic drug used for the treatment of advanced stage ACC. We have also shown that FATE1 expression counteracts cell death induced by agents stimulating ER-mitochondria Ca^{2+} transfer. On the other hand, we could not follow the interesting suggestion of this reviewer about expressing the BIK protein in our cell lines since their transfection efficiency is too poor to perform downstream batch assays like caspase 3/7 activation. However, interestingly enough, while our paper was under revision a study was published (cited as ref. 42 in our manuscript) confirming our results about FATE1 having an anti-apoptotic function in a variety of cancer cell lines (not including ACC) and showing interaction between FATE1 and BIK, even if only in transiently transfected cells.

The sample numbers in Fig 6b for non-steroidogenic tumors are too low and thus not very meaningful.

We have now analyzed FATE1 expression in a series of 77 cancer samples of various histotypes. None of them was positive for FATE1 expression. These results are shown in Fig. 5B of the revised manuscript.

Referee #3

In their manuscript "FATE1 antagonizes calcium- and drug-induced apoptosis by uncoupling ER and mitochondria" Doghman et al. describe their analysis on the cancer-testis antigen encoded by FATE1. Based on the performed experiments the authors conclude that the FATE1 protein resides in the outer mitochondrial membrane and localizes to mitochondria-associated-membranes (MAM) that represent close contacts between the ER and mitochondria. Furthermore, the authors conclude that varying FATE1 levels impact the distance between ER and mitochondria, which has consequences for mitochondrial Ca2+-import and the sensitivity to mitochondrial Ca2+-dependent proapoptotic stimuli.

ER-mitochondria interfaces have in recent years emerged as important cellular structures that have been implicated in mitochondrial fission and calcium and lipid transport between the ER and mitochondria. Given the importance of these processes for cellular function, the identification of the structural and regulatory components of ER-mitochondria interfaces is of outstanding cell biological interest. Therefore, the identification of FATE1 as a regulatory component of ER-mitochondria interfaces would be a very valuable and important contribution.

This manuscript is well written and the data are of good quality. The authors conclude based on their data that FATE1 is a component that regulates ER-mitochondria interfaces. This conclusion hinges on the co-immunoprecipitation experiments identifying ER-resident proteins as interacting proteins of FATE1, the subcellular fractionation study that identifies FATE1 as a component of the MAM fraction and the microscopic analyses on ER-mitochondria interfaces that suggest that these interfaces are modulated dependent on FATE1 levels. However at this point, the provided data do not sufficiently support the conclusions drawn.

1. It is interesting that MAM-enriched proteins are co-immunoprecipitated with FATE1; however, the data provided in the current manuscript make it impossible to evaluate the degree of interaction. It is difficult to assess, whether any controls have been performed (pull-down experiment in an untransfected cell-line) that show that the interactions are specific. Given the importance of a physical interaction of FATE1 and ER-resident proteins for the conclusion,

reciprocal pull-down experiments with FATE1 and other MAM components would likewise be greatly supportive. Such experiments should be included in the main part of the manuscript.

We thank this reviewer for his/her positive appreciation of our study. As also detailed in our answer to point 3) raised by Referee #1, in the revised version of our manuscript we show the results of a coimmunoprecipitation experiment performed in Dox-treated H295R/TR N-Flag FATE1 cells (stable Dox-inducible clone expressing moderate amounts of FATE1 after Dox treatment) where FATE1 is found to interact specifically with the ER and MAM-localized protein EMD, one of the FATE1-interacting partners we identified by mass spectrometry (Fig. 2C). In addition, FATE1 – EMD interaction is confirmed by data present in databases from high-throughput yeast two-hybrid screening campaigns (ref. 17 in our manuscript).

2. The subcellular fractionation studies in Figure 3a are interpreted to suggest that FATE1 localizes into MAMs. It is, however, puzzling that in this experiment FATE1 is enriched in the ER fraction, compared to the 'crude mitochondrial' fraction. This is at odds with the microscopy data provided in Figure 1d, 1e and many parts of Figure 2 that show that the majority of FATE1 is at the OMM. Furthermore, Figure 3a shows that FATE1 is markedly dis-enriched from the purified mitochondrial fraction. The data presented in 3a suggests that the majority of FATE1 localizes to the ER and is enriched at ER-mitochondria interfaces. These results are difficult to reconcile with the microscopy data that show a broad distribution of FATE1 across the whole mitochondrial organelle and not a focal localization to ER-mitochondria contact sites. These discrepancies raise doubts about the subcellular fractionation experiments and the microscopy data and must be experimentally clarified. A triple
labeling experiment examining mitochondria, ER and FATE1 localization might more strongly support the proposed enrichment of FATE1 at ER-mitochondria interfaces.

As also detailed in our answer to point 1) raised by Referee #1, our interpretation of the apparently conflicting morphological and biochemical results about FATE1 localization is that during the MAM purification procedure FATE1 may have been "stripped" from the OMM due to tight interactions with MAM and ER proteins (including EMD, which is highly enriched in MAM as shown in Fig. 2E). We have discussed our findings in the Discussion section of our manuscript at page 12. However, we would like to underline again here that the same cell type (Dox-treated H295R/TR SF-1 cells) was used to assess endogenous FATE1 subcellular localization using both biochemical and morphological methods and that MAM preparation was performed using a well-established method (see ref. 48 in our paper) and repeated several times in our laboratory, always producing the same results, as shown in Fig. 2E. Furthermore, control proteins known to be enriched in the MAM fraction (SERCA2, S1R, VDAC) and a mitochondrial OMM protein (TOM20) show the respective expected localization after our MAM preparation procedure.
Furthermore, following the interesting suggestion of this reviewer, we have added to our manuscript triple fluorescence localization results in Dox-treated H295R/TR SF-1 (Fig. 2F and Appendix Fig. S2), H295R/TR N-Flag FATE1 (Appendix Fig. S3) and in HeLa cells transiently transfected with a FATE1 expression vector (Appendix Fig. S4). In all images, colocalization of FATE1, ER and mitochondria signals can be observed, as shown by the images and by their curve colocalization profiles.

3. The data provided in Figure 3b are interpreted to suggest that ER-mitochondrial distance increases in response to FATE1 overexpression. Light fluorescence microscopy does not have the spatial resolution necessary to clearly distinguish between ER and mitochondria that are in the vicinity of one another and actual contact sites between both organelles. At this point it is entirely possible that subtle changes in organelle morphology, which could be a result of FATE overexpression, would result in the same difference in colocalization of mitochondria and ER determined by this method. The authors try to support these data by utilizing a split-GFP system, in which complementing parts of GFP are targeted to the mitochondrial outer membrane and the ER. This is a very interesting assay; however, by following the provided reference, no evidence can be found that sufficiently validates this method, specifically for ER-mitochondria interfaces. Therefore, concluding altered

ER-mitochondria distance based on this assay seems premature. Further support for altered ER-mitochondria contacts dependent on FATE1 levels must be provided. Such experiments could for example utilize microscopy techniques with higher spatial resolution.

As also detailed in our answer to point 5) raised by Referee #1, in the revised version of our manuscript we have added the results of EM quantification of ER-mitochondria contacts in basal conditions and after Dox treatment both in H295R/TR N-Flag FATE1 and H295R/TR SF-1 cells. Both the number of ER-mitochondria contacts and the number of mitochondria displaying ER contact sites were taken into account (Fig. 3C, D). The EM data confirmed the results found using the two other approaches, that is that FATE1 expression decreases ER-mitochondria interaction.

In summary, while this manuscript attempts to assign FATE1 a direct role in mitochondrial-ER attachment site regulation, further experiments must be done to support this conclusion. Without such experiments the conclusion that FATE1 affects mitochondrial Ca2+-import and the sensitivity to mitochondrial Ca2+-dependent proapoptotic stimuli by modulating ER-mitochondria contacts still remains too speculative and could as well be explained by FATE1 levels broadly affecting other aspects of mitochondrial biology.

The link beween the plasticity of ER-mitochondria tethering and regulation of mitochondrial shape is obviously complex. However, we believe that in our manuscript we have provided solid experimental evidence for FATE1 being implicated in the regulation of ER-mitochondria contacts.

Other comments:

- *Enlarged regions in the microscopy data in Figure 1 should be provided to allow assessment of the localization of FATE1 relative to the other markers.*

We show microscopy images with higher enlargements in the revised version of our manuscript (Fig. 1D, E – Fig. 2B, F – Fig. 3B – Figs. EV1 and EV2 – Appendix Figures S2-S4).

- *In Figure 2, a panel needs to be added to schematically illustrate the mutants used in the study.*

We have added a scheme of the FATE1 protein in Fig. 2A. Since this is a very busy figure, no space was left to add a panel illustrating the different FATE1 mutants used in our study, as suggested by this reviewer. However, the precise nature and boundaries of the mutants are clearly indicated in Fig. 2B, Fig. EV1 and EV2.

- *In Figure 3b, enlarged regions need to be shown.*

We show a higher magnification for GFP signals in Fig. 3B in the revised manuscript.

- *Why is mt-CA2+ content lower in cells transfected with n-Flag FATE1? This needs to be explained and discussed.*

It is common experience that both basal and stimulated mitochondrial Ca^{2+} levels may vary even between similar cell lines. It is important to underline here that H295R/TR N-Flag FATE1 cells are not transiently transfected but express moderate amounts of N-terminally tagged FATE1 under the control of a Dox-inducible integrated transgene (Fig. 1B).

- *Figure 4 should be moved to the supplement. Also, a quantification of the detected signals might support the authors' claims, as small differences in the levels can be observed for some of the analyzed proteins. A figure for the co-immunoprecipitation data should be included in the main part of the manuscript.*

As suggested by this reviewer, that immunoblot has been moved to Fig. EV4 in the revised version of our manuscript and protein signals quantified using the ImageJ software after normalization by b-tubulin levels. Coimmunoprecipitation data are shown in Fig. 2C.

2nd Editorial Decision 30 March 2016

Many thanks for the submission of your revised study to EMBO reports and for your patience while we were waiting to hear back from the last referee whose feedback we have just now received.

As you will see, while referee 2 is satisfied with the way in which you have addressed his/her concerns, both referees 1 and 3 still raise a number of, mostly overlapping, issues. First of all, both of them again raise the issue of the discrepancy between FATE localization in biochemical and immuno-fluorescence experiments. In essence, neither of them is convinced that the data as presented is strong enough to conclusively prove that FATE1 localizes to mitochondria and that it acts at ER-mitochondrial contact sites in the proposed way. However, reviewer 3 suggests some potential ways of strengthening these data and since this was brought up by two reviewers in two rounds of review, we would have to insist on the addition of further experiments to address this issue, rather than toning down or more carefully discussing the problems. Please refer to the reports for the detailed suggestions of the reviewers on how to improve the data. With regard to the issue raised by reviewer 1 on the functional interaction of FATE1 with ER-localized emerin, we think it would be sufficient to more carefully discuss a potential role for this protein in the FATE1-mediated regulation of ER-mitochondrial contacts.

Given that both referees 1 and 3 in principle agree on the potential interest of your study, we would like to give you the exceptional opportunity to revise your manuscript again, with the understanding that the remaining issues of the reviewers need to be addressed in the final version of the manuscript.

Formally, papers in EMBO reports have to be accepted within 6 months of the initial decision, which in your case would be May 8th, 2016. We are, of course, still interested in publishing your study after this date, but we would need to take the novelty into account if your study can only be accepted after this date.

While looking at the figures, I also noticed that there seem to be some spliced/cropped lanes in some western blots and as it is our journal's policy in such cases, authors have to present raw data for all experiments (i.e. original, uncropped western blots etc.) to our editorial office. Please do include these data when submitting your final version. In addition, in cases, where splicing was done, please clearly indicate these events through black or white vertical lines.

I look forward to seeing the final version of the study as soon as possible and remain with kind regards

REFEREE REPORTS

Referee #1:

This manuscript addresses the function of a cancer testes antigen FATE1 in regulating ER/mitochondrial contacts and calcium flux. The overall idea is that genes normally expressed only in gametes, become expressed in somatic cells in a process that contributes to the tumorigencity. Therefore, why or whether expression of FATE1 promotes cancer is important as it may represent a therapeutic target in immunotherapy or other approaches. I had a number of critical concerns with the first submission of the manuscript, and many have been addressed in revision. The authors have clarified some points, and shown that steroid production is also regulated by FATE1 (reflecting it's role in the adrenergic cells), they validated the co-IP with EMD, and they tried to quantify mitochondrial morphological changes upon manipulation of FATE1. I appreciate the effort made by the authors for the revision.

Importantly, another study was published in Nature Communications in the meantime (as briefly

cited by the authors in the discussion), which I think can really complement the work presented here. In it (Nat Commun. 2015 Nov 16;6:8840) those authors interrogated a number of tumor lines expression CTAs and examined the contribution of each protein to survival, signaling and proliferation. They focused on FATE1, which showed the most robust protection against cell death - as silencing FATE1 led to the dramatic induction of apoptosis. This was through the recruitment of a specific ubiquitin E3 ligase to degrade the anti-apoptotic protein Bik. Their work clearly defines a role for FATE1 in cell death, providing meaningful mechanism. However, the complementarity to the current work lies in the previous identification of Bik's role in promoting cell death through ER contacts with mitochondria, and in the well established requirement for calcium flux during cell death. The work here indicates that loss of FATE1 leads to a reduced number of ER contact sites, and reduced calcium uptake into mitochondria. They observe a very mild protection against stimulated cell death, which is perhaps not consistent with the more robust survival activity of FATE1 seen in the Maxfield et al study. This was also a concern in my previous review - that the effects on caspase activation were mild. But I agree there is a trend, and this effect is likely dependent on the cancer line being analyzed.

I am supportive of the work to better characterize the function of FATE1, as it is obviously of importance, and seems to have a role in regulating the apoptotic machinery at the ER/mitochondrial contact sites, which promotes survival in the cancer (and testes) paradigm. However, I have 3 major concerns/suggestions that the authors need to address.

1. My primary concern was the discrepancy between the biochemistry and IF localization of FATE1, which was also the concern of Reviewer 3. The fractionation in Figure 2 clearly shows FATE1 as a primarily ER protein, with some smaller fraction in the MAM. In response to my concerns the authors state: "We interpret these apparently conflicting results in the sense that during the MAM purification procedure tight interactions of FATE1 with MAM and ER proteins (including EMD, which is highly enriched in MAM as shown in Fig. 2E) can "strip" it from the mitochondrial outer membrane, to which the protein is associated through a short C-terminal transmembrane domain." This is a speculative explanation that does not address the discrepancy. The explanation they have would represent a completely unexpected biochemical behavior of a transmembrane spanning protein essentially being solubilized during a fractionation experiment. Does FATE1 actually have a functional transmembrane domain? It is mechanistically very important that we know whether FATE1 is mitochondrial or ER. I offer a suggestion based on the data shown in this submission and the recently published Nat Comm paper, which also looked at the localization. In my opinion, the C-terminal TMD is targeting the protein primarily to the ER (as seen from their TMD construct), and the coiled coil domain by the L151 residue may tightly engage a mitochondrial protein(s). Losing the coiled coil (Nat Comm paper), or mutating the L151 residue (here) resulted in an ER localization. Clearly it is unprecedented to have an ER "domain" so closely aligned with the mitochondria that IF and gold would suggest a mitochondrial protein. The fractionation could easily lead to the disruption of coiled coil mediated interactions, revealing the ER/MAM localization of FATE1. This explanation makes more sense than assuming a complete extraction "stripping" of a membrane anchored domain. I do not doubt the technical abilities of the authors - the data is the data - but it is so very unusual that I don't agree with the explanation given by the authors without further evidence. At a minimum they must admit to this discrepancy more seriously, and perhaps offer a few different explanations, including what I have raised.

2. These authors focused on emerin (EMD) as an interacting partner. In the first submission this interaction was not validated and the interaction was not shown to be functionally required for the phenotypes they were following. Both myself and reviewer 3 focused on this inadequacy in the mansuscript. In the revision the interaction was validated with IP - and the interaction was seen by others in 2 hybrid screens. With this simple IP, they draw a model that depicts a clear requirement for this interaction in the regulation of ER contact sites, however any role for emerin in this process was absolutely not demonstrated here, and the model cannot stand as it is. In this concern, the authors have fallen short to convince me that this interaction is functionally important.

3. The third issue of major importance is to put this work in context of the recent Nature Communications paper. Indeed, the great strength of the Nat Comm paper was the identification of the E3 ligase that binds FATE1 and targets Bik, providing an explanation for the survival function of FATE1. It is clear that Bik acts from the ER, and drives apoptosis through ER stress and calcium flux. That Bik is the selective target of FATE1 is extremely interesting and the links to calcium

made here strengthen this I think. The increased calcium uptake in siFATE1 cells is consistent with an accumulation of Bik. Therefore the authors should consider this information more seriously in interpreting the data presented here.

4. Last, more minor point: The EM images showing the ER/contacts should have a higher magnification so we can appreciate the distances - are they 20nm or ~100? There may be extended contacts with larger distances?

Referee #2:

The authors have addressed my concerns, I am satisfied with the revision.
This is a very interesting paper.

Referee #3:

Doghman et al. present a revised version of their manuscript entitled "FATE1 antagonizes calcium- and drug-induced apoptosis by uncoupling ER and mitochondria". The authors address the concerns of the reviewers and to this end have performed several new experiments to support their conclusion that FATE1 has an important role in regulation of ER-mitochondria distance and Ca2+ uptake by mitochondria.

The manuscript is very interesting and in my opinion convincingly demonstrates that FATE1 is a protein localized to the outer mitochondrial membrane, that it interacts with the ER-localized protein EMD, that altering the expression levels of FATE1 leads to alteration in mitochondrial morphology, number of contact site between mitochondria and the ER and Ca2+ uptake by mitochondria. In my opinion it is not satisfyingly demonstrated that FATE1 acts directly at mitochondria-ER contact sites. Furthermore, the question of whether the phenotypes observed upon altering expression levels of FATE1 are due to a direct role of FATE1 at mitochondria-ER contact sites or are indirect effects of a different function of FATE1 (e. g. altering mitochondrial morphology) is at this point inconclusively resolved.

Below are the responses to the specific concerns raised in our initial review, that were addressed by the authors:
1. It is interesting that MAM-enriched proteins are co-immunoprecipitated with FATE1; however, the data provided in the current manuscript make it impossible to evaluate the degree of interaction. It is difficult to assess, whether any controls have been performed (pull-down experiment in an untransfected cell-line) that show that the interactions are specific. Given the importance of a physical interaction of FATE1 and ER-resident proteins for the conclusion, reciprocal pull-down experiments with FATE1 and other MAM components would likewise be greatly supportive. Such experiments should be included in the main part of the manuscript.

The additional pull-down experiments convincingly demonstrate a physical interaction between FATE1 and EMD. Since the presented microscopy experiments and the subcellular fractionation studies support a localization of EMD in the ER, an interaction between the mitochondrially localized FATE1 with EMD is supportive of a role of FATE in regulating mitochondria-ER contacts. Because very little is know about the role of EMD at the ER, direct roles of FATE1 and EMD at mitochondria-ER interfaces should be discussed very carefully.

2. The subcellular fractionation studies in Figure 3a are interpreted to suggest that FATE1 localizes into MAMs. It is, however, puzzling that in this experiment FATE1 is enriched in the ER fraction, compared to the 'crude mitochondrial' fraction. This is at odds with the microscopy data provided in Figure 1d, 1e and many parts of Figure 2 that show that the majority of FATE1 is at the OMM. Furthermore, Figure 3a shows that FATE1 is markedly dis-enriched from the purified mitochondrial fraction. The data presented in 3a suggests that the majority of FATE1 localizes to the ER and is enriched at ER-mitochondria interfaces. These results are difficult to reconcile with the microscopy data that show a broad distribution of FATE1 across the whole mitochondrial organelle and not a focal localization to ER-mitochondria contact sites. These discrepancies raise doubts about the subcellular fractionation experiments and the microscopy data and must be experimentally clarified.

A triple labeling experiment examining mitochondria, ER and FATE1 localization might more strongly support the proposed enrichment of FATE1 at ER-mitochondria interfaces.

The subcellular fractionation studies are still of concern. I have no doubts that the authors have reproduced these data and I also understand that the MAM purification procedure follows established protocols. This, however, does not bring the conflicting results obtained by microscopy and subcellular fractionation into accordance. From the data presented by the authors it is evident that the subcellular fractionation is prone to artifacts, since FATE1 is removed from the mitochondrial fraction. Another concern for the subcellular fraction studies is the result obtained for GRP75. In the text the authors introduce GRP75 as an "ER-chaperone [....], which is known to be enriched in MAM structures". Their fractionation studies, however, show that GRP75 is recovered in the mitochondrial fraction (PM) and is, at best, very slightly enriched in MAM's, which therefore raises further doubts in the subcellular fractionation experiment. Given these concerns, a localization of FATE1 at mitochondria-ER interfaces has to be supported by alternative approaches such as microscopy-based approaches. To this end, the authors provide an experiment in their revised manuscript, in which they use triple labeling of FATE1, mitochondria and ER in fluorescence microscopy studies. Such an experiment could potentially support an enrichment of FATE1 at mitochondria-ER contact sites. The provided figure is, however, not interpretable. The provided figure shows enlarged regions of the cell that are very crowded and it is impossible to assess, whether FATE1 is enriched at mitochondria in regions where mitochondria and the ER are in close proximity to one another. Also the quantification provided in the appendix does not provide this information, it merely shows that FATE1 is present on mitochondria also at sites where the ER is, but not that it is enriched at these sites. To improve this figure, the authors could choose regions of a cell that are less crowded for their analysis, which could potentially support enrichment of FATE1 at mitochondria-ER contact sites. In its current state this figure (Figure 2F) is of no value and should be omitted if it can't be improved.
In conclusion, it is speculative to conclude that FATE1 is localized to MAM's and the provided data have to be interpreted very carefully. Furthermore, it has to be clearly stated in the results section that the results obtained by the subcellular fractionation study and microscopy are conflicting.

3. The data provided in Figure 3b are interpreted to suggest that ER-mitochondrial distance increases in response to FATE1 overexpression. Light fluorescence microscopy does not have the spatial resolution necessary to clearly distinguish between ER and mitochondria that are in the vicinity of one another and actual contact sites between both organelles. At this point it is entirely possible that subtle changes in organelle morphology, which could be a result of FATE overexpression, would result in the same difference in co-localization of mitochondria and ER determined by this method. The authors try to support these data by utilizing a split-GFP system, in which complementing parts of GFP are targeted to the mitochondrial outer membrane and the ER. This is a very interesting assay; however, by following the provided reference, no evidence can be found that sufficiently validates this method, specifically for ER-mitochondria interfaces. Therefore, concluding altered ER-mitochondria distance based on this assay seems premature. Further support for altered ER-mitochondria contacts dependent on FATE1 levels must be provided. Such experiments could for example utilize microscopy techniques with higher spatial resolution.

In the revised version of their manuscript the authors include EM experiments to support data obtained by fluorescence microscopy that indicate that the FATE1 overexpression leads to a reduced number of contact sites between mitochondria and ER. The EM data convincingly demonstrate that FATE1 overexpression leads to changes in the observed number of contact points between mitochondria and the ER.

The fluorescence microscopy data presented in figure 3a are very difficult to interpret, because the resolution of conventional light fluorescence microscopy has limiting resolving power to distinguish between ER and mitochondria that are in the vicinity of one another and actual contact sites between both organelles. Especially the shown magnification and quality of the image does not allow the reader to assess the degree of co-localization of mitochondria and ER. Images of higher quality and enlarged regions have to be presented.

The split-GFP is an interesting assay, but, as mentioned previously, at no point has this assay been validated and it is not clear to me, whether the GFP signal reports faithfully on mitochondria-ER

contact sites. If this experiment is included in the manuscript the assay has to be validated in some form.

2nd Revision - authors' response 30 April 2016

We appreciate the positive feedback by all three reviewers and are grateful for the comments we received on the previous version of our manuscript that helped us to further improve it. In the new revised version of our manuscript we were able to address all criticisms that the reviewers still had. Here follows a detailed, point-by-point rebuttal to the reviewers' comments and criticisms:

Referee #1
This Reviewer appreciated the efforts we made to improve the previous version of our manuscript and is overall supportive of our work. However, s/he expressed three major and one minor concerns that we believe we addressed in full in the new version of our manuscript, as explained here below:

1) My primary concern was the discrepancy between the biochemistry and IF localization of FATE1, which was also the concern of Reviewer 3. The fractionation in Figure 2 clearly shows FATE1 as a primarily ER protein, with some smaller fraction in the MAM. In response to my concerns the authors state: "We interpret these apparently conflicting results in the sense that during the MAM purification procedure tight interactions of FATE1 with MAM and ER proteins (including EMD, which is highly enriched in MAM as shown in Fig. 2E) can "strip" it from the mitochondrial outer membrane, to which the protein is associated through a short C-terminal transmembrane domain." This is a speculative explanation that does not address the discrepancy. The explanation they have would represent a completely unexpected biochemical behavior of a transmembrane spanning protein essentially being solubilized during a fractionation experiment. Does FATE1 actually have a functional transmembrane domain? It is mechanistically very important that we know whether FATE1 is mitochondrial or ER. I offer a suggestion based on the data shown in this submission and the recently published Nat Comm paper, which also looked at the localization. In my opinion, the C-terminal TMD is targeting the protein primarily to the ER (as seen from their TMD construct), and the coiled coil domain by the L151 residue may tightly engage a mitochondrial protein(s). Losing the coiled coil (Nat Comm paper), or mutating the L151 residue (here) resulted in an ER localization. Clearly it is unprecedented to have an ER "domain" so closely aligned with the mitochondria that IF and gold would suggest a mitochondrial protein. The fractionation could easily lead to the disruption of coiled coil mediated interactions, revealing the ER/MAM localization of FATE1. This explanation makes more sense than assuming a complete extraction "stripping" of a membrane anchored domain. I do not doubt the technical abilities of the authors - the data is the data - but it is so very unusual that I don't agree with the explanation given by the authors without further evidence. At a minimum they must admit to this discrepancy more seriously, and perhaps offer a few different explanations, including what I have raised.
We thank this Reviewer very much for this comment and for the suggestions s/he made. We believe s/he is entirely right in the statement that it is difficult to conceive that biochemical fractionation procedures may extract a membrane protein from the membranes where it is localized without the use of detergents, as we initially suggested. Following his/her suggestion, we have added to the manuscript more detailed data showing that indeed the mutant FATE1 EGFP fusion proteins L151D and isolated transmembrane domain (TMD) have sigificantly enhanced colocalization with ER and decreased colocalization with mitochondria compared to the wild-type protein. These data are shown in Fig. EV2 and are consistent with results shown in the paper by Maxfield *et al.* (ref. 53 in our manuscript) mentioned by this reviewer. We then share with this Reviewer the opinion that the FATE1 transmembrane domain is likely to be inserted in the ER membrane (as shown in Fig. 2H) and that interaction with mitochondrial partners mediated by the C-terminal coiled coil domain of the protein are required for mitochondrial localization of FATE1. We have identified by mass spectrometry one of these mitochondrial FATE1 interacting-proteins as Mic60/mitofilin. FATE1 – Mic60 interaction was confirmed by coimmunoprecipitation (Fig. 2E). As pointed out by the Reviewer, this is a very unusual situation that has no antecedents to our knowledge. Importantly, this new interpretation of our data allows to reconcile the discrepancy between the results concerning subcellular localization and biochemical fractionation of FATE1. In the light of these results, we have changed the text of our manuscript in the Results section at pages 5, 6 and 8 and in

the Discussion section at pages 13-14. We have also accordingly modified our model of FATE1 function (Fig. 5E).

2) These authors focused on emerin (EMD) as an interacting partner. In the first submission this interaction was not validated and the interaction was not shown to be functionally required for the phenotypes they were following. Both myself and reviewer 3 focused on this inadequacy in the mansuscript. In the revision the interaction was validated with IP - and the interaction was seen by others in 2 hybrid screens. With this simple IP, they draw a model that depicts a clear requirement for this interaction in the regulation of ER contact sites, however any role for emerin in this process was absolutely not demonstrated here, and the model cannot stand as it is. In this concern, the authors have fallen short to convince me that this interaction is functionally important.
This is another well taken comment from this Reviewer. It is true that we have no direct evidence about the role of emerin in the regulation of ER-mitochondria contact sites. However, it is remarkable that for the first time we have shown that EMD is enriched in MAM (Figure 2H). This finding may open new scenarios for the understanding of the biological function of this protein that has a direct role in human disease. We then believe it is important that these data will be published since they will concern all scientists with an interest in EMD and in its associated disease. In compliance with the comments of this Reviewer, in the Discussion section of the newly revised version of our manuscript we have briefly resumed our findings and stated that "further studies are needed to assess the role of EMD in ER-mitochondria communication and its potential impact on the pathogenesis of EDMD" (page 14).

3) The third issue of major importance is to put this work in context of the recent Nature Communications paper. Indeed, the great strength of the Nat Comm paper was the identification of the E3 ligase that binds FATE1 and targets Bik, providing an explanation for the survival function of FATE1. It is clear that Bik acts from the ER, and drives apoptosis through ER stress and calcium flux. That Bik is the selective target of FATE1 is extremely interesting and the links to calcium made here strengthen this I think. The increased calcium uptake in siFATE1 cells is consistent with an accumulation of Bik. Therefore the authors should consider this information more seriously in interpreting the data presented here.
We have cited as ref. 53 in our manuscript the recent paper by Maxfield *et al.* showing destabilization of BIK expression by FATE1 in a variety of cancer cell lines. However, employing the same antibody used in the work by Maxfield *et al*., we have shown in our newly revised paper that BIK expression was not modulated by FATE1 in any of the H295R-derived cell lines used in our study (Appendix Fig. S6). These data suggest that in adrenocortical carcinoma (ACC) cells FATE1 anti-apoptotic action is not dependent on modulation of BIK expression. Further studies are required to assess whether FATE1 interacts with BIK also in H295R cells. We did not find BIK among the FATE1 interactors we identified by mass spectrometry (Appendix Table S1). This finding of course does not rule out the possibility that BIK may interact with FATE1 also in ACC cells, but we would like to point out here that FATE1-BIK (and RNF183) interactions reported in the paper by Maxfield *et al.* were only shown in transiently transfected cells. Furthermore, no direct evidence is shown in that paper that RNF183 ubiquitinates BIK targeting it to destruction, but only that RNF183 overexpression resulted in BIK downregulation. Taking into account the results shown both in the Maxfield *et al.* and in our own studies, there is no doubt that further investigations are required to fully elucidate the fascinating issue of the mechanisms used by FATE1 to regulate apoptosis in different types of cancer.

4) Last, more minor point: The EM images showing the ER/contacts should have a higher magnification so we can appreciate the distances - are they 20nm or ~100? There may be extended contacts with larger distances?
As suggested by this Reviewer, in the new revised version of our manuscript we show EM images with higher magnifications (Fig. 3C, D). We focused our analysis of ER-mitochondria interactions to close (<50 nm) contacts.

Referee #2
We are glad that this Reviewer appreciated the relevance of our study and supported its publication.

Referee #3

We thank this Reviewer for his/her thorough analysis of our results and for appreciating the importance of our study. Here are our detailed replies to the remaining concerns expressed by this Reviewer:

1) The additional pull-down experiments convincingly demonstrate a physical interaction between FATE1 and EMD. Since the presented microscopy experiments and the subcellular fractionation studies support a localization of EMD in the ER, an interaction between the mitochondrially localized FATE1 with EMD is supportive of a role of FATE in regulating mitochondria-ER contacts. Because very little is know about the role of EMD at the ER, direct roles of FATE1 and EMD at mitochondria-ER interfaces should be discussed very carefully.

See our reply to the observations made by Reviewer #1, point 2.

2) The subcellular fractionation studies are still of concern. I have no doubts that the authors have reproduced these data and I also understand that the MAM purification procedure follows established protocols. This, however, does not bring the conflicting results obtained by microscopy and subcellular fractionation into accordance. From the data presented by the authors it is evident that the subcellular fractionation is prone to artifacts, since FATE1 is removed from the mitochondrial fraction. Another concern for the subcellular fraction studies is the result obtained for GRP75. In the text the authors introduce GRP75 as an "ER-chaperone [....], which is known to be enriched in MAM structures". Their fractionation studies, however, show that GRP75 is recovered in the mitochondrial fraction (PM) and is, at best, very slightly enriched in MAM's, which therefore raises further doubts in the subcellular fractionation experiment. Given these concerns, a localization of FATE1 at mitochondria-ER interfaces has to be supported by alternative approaches such as microscopy-based approaches. To this end, the authors provide an experiment in their revised manuscript, in which they use triple labeling of FATE1, mitochondria and ER in fluorescence microscopy studies. Such an experiment could potentially support an enrichment of FATE1 at mitochondria-ER contact sites. The provided figure is, however, not interpretable. The provided figure shows enlarged regions of the cell that are very crowded and it is impossible to assess, whether FATE1 is enriched at mitochondria in regions where mitochondria and the ER are in close proximity to one another. Also the quantification provided in the appendix does not provide this information, it merely shows that FATE1 is present on mitochondria also at sites where the ER is, but not that it is enriched at these sites. To improve this figure, the authors could choose regions of a cell that are less crowded for their analysis, which could potentially support enrichment of FATE1 at mitochondria-ER contact sites. In its current state this figure (Figure 2F) is of no value and should be omitted if it can't be improved. In conclusion, it is speculative to conclude that FATE1 is localized to MAM's and the provided data have to be interpreted very carefully. Furthermore, it has to be clearly stated in the results section that the results obtained by the subcellular fractionation study and microscopy are conflicting.

This is a crucial point raised by this Reviewer, similar to point 1) by Reviewer #1. As we explained previously in our rebuttal to that point, we believe that, as suggested by Reviewer #1, the data shown in Fig. 2 and Fig. EV3 provide evidence that the TMD domain of FATE1 localizes in the ER, while the mitochondrial localization of the protein is determined by interaction with partner(s) which is impaired by the L151D mutation in the FATE1 C-terminal coiled coil domain. In addition to these morphological data, localization of FATE1 at the interface between ER and mitochondria is confirmed by coimmunoprecipation and biochemical fractionation results. Remarkably, FATE1, which is part of a very high molecular weight complex (Appendix Fig. S3), can be coimmunoprecipitated both with ER-resident (EMD) and mitochondrial (Mic60/mitofilin) proteins (Fig. 2C, E). A robust and widely used procedure for MAM preparation reproducibly showed that FATE1 fractionates in crude mitochondria, ER and MAM, but not in the pure mitochondrial fraction (Fig. 2H). Reliability of the procedure is shown by the expected fractionation of ER, mitochondrial and MAM markers in those experiments. The localization of GRP75 in our experiments mentioned by this Reviewer is actually consistent with previous data showing that this chaperone fractioned prevalently in the pure mitochondrial fraction on top of also being present in MAM (ref. 28 in our manuscript). We apologize for any misunderstanding that the inexact text formulation in the previous version of our manuscript may have induced. We have therefore modified the text of our manuscript at page 8. Because of the criticisms expressed by this Reviewer, we have also replaced the triple labelling immunofluorescence image of a Dox-treated H295R/TR SF-1 in the previous version of our manuscript with a new one (Fig. 2G and Appendix Fig. S4)

showing colocalization of endogenous FATE1, mitochondrial marker HSP60 and ER marker calreticulin at various sites. The same data are shown for Dox-treated H295R/TR N-Flag FATE1 cells in Appendix Fig. S5. Altogether, we are convinced that the morphological and biochemical data shown in our manuscript provide compelling evidence for presence of FATE1 in ER-mitochondria contact sites, which explains its effect on trans-organelle calcium transfer and modulation of apoptosis.

3) In the revised version of their manuscript the authors include EM experiments to support data obtained by fluorescence microscopy that indicate that the FATE1 overexpression leads to a reduced number of contact sites between mitochondria and ER. The EM data convincingly demonstrate that FATE1 overexpression leads to changes in the observed number of contact points between mitochondria and the ER.
The fluorescence microscopy data presented in figure 3a are very difficult to interpret, because the resolution of conventional light fluorescence microscopy has limiting resolving power to distinguish between ER and mitochondria that are in the vicinity of one another and actual contact sites between both organelles. Especially the shown magnification and quality of the image does not allow the reader to assess the degree of co-localization of mitochondria and ER. Images of higher quality and enlarged regions have to be presented. The split-GFP is an interesting assay, but, as mentioned previously, at no point has this assay been validated and it is not clear to me, whether the GFP signal reports faithfully on mitochondria-ER contact sites. If this experiment is included in the manuscript the assay has to be validated in some form.
In compliance with the criticisms by this Reviewer, we now show better resolution images in Fig. 3A of our manuscript where modulation of the extension of ER-mitochondria contact sites upon FATE1 expression is displayed. Data were quantified by analysis of confocal stacks in multiple cells and results are presented in the histogram shown in Fig. 3A, which shows a significant decrease of ER-mitochondria colocalization following FATE1 expresssion. Concerning the other point raised by this Reviewer, we would like to mention that the split-GFP assay used in our study has recently been used to assay ER-mitochondria distance in a very recent publication from Prof. Rizzuto's group (ref. 29 in our manuscript). In that study, the authors showed that fibroblasts from patients carrying the 13514A>G mutation in the ND5 subunit of NADH dehydrogenase display an increased autophagic flux due to downregulation of mitochondrial Ca^{2+} uptake. This correlated with decreased contact between ER and mitochondria measured by the same assay used in our study. To validate the system, the ER-mitochondria tethering factor Mfn2 was overexpressed in patients' fibroblasts. This led to a significant increase in the number of contact sites in those cells, as measured by the split-GFP assay (Figure 1).

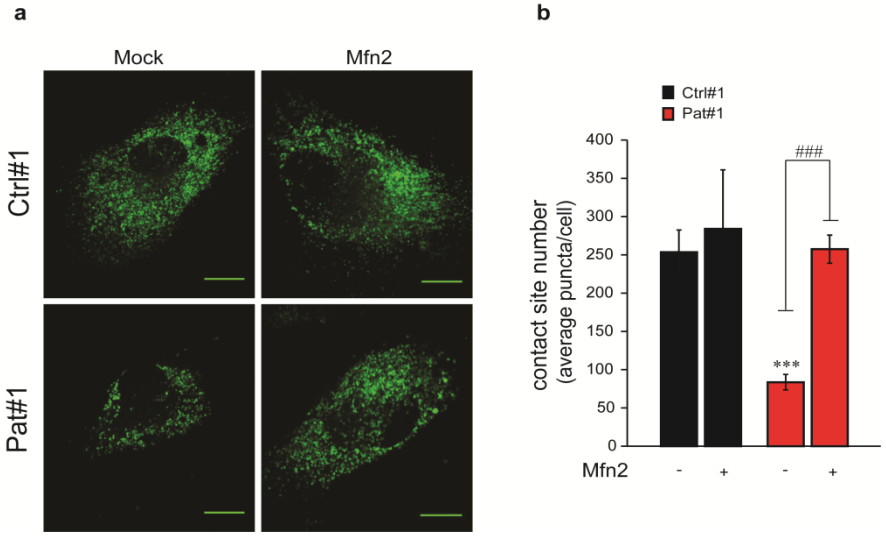

Figure 1. (**a**) Healthy donor- (Ctrl#1) and 13514A>G patient-derived (Pat#1) fibroblasts were co-transfected with an OMM anchored GFP lacking an essential beta strand (GFP 1-10) and an ER-

anchored beta strand 11 required for GFP1-10 fluorescence reconstitution upon complementation and Mfn2 where indicated. After 24 hours, cells were fixed and imaged. Scale bars represent 10μm. (**b**) Quantification of contact sites between ER and mitochondria of cells processed as in panel **a**. ***p<0.0001 compared to control. ###p<0.0001 between the indicated groups.

Finally and most importantly, we would like to underline that the data obtained using three independent techniques (fluorescence confocal microscopy, split-GFP assay and EM) all converge to indicate that consequently to FATE1 expression ER-mitochondria contact sites significantly decrease in ACC cells.

3rd Editorial Decision 31 May 2016

Thank you for submitting your revised manuscript to EMBO reports and for your patience while we were waiting for the feedback from the reviewers.

In principle, both referees now support publication of your study here, but referee 3 remains unconvinced about the FATE1 localization.

S/he suggests to more carefully discuss this issue and also proposes a - in my opinion - rather straightforward way to address this concern analytically.

Therefore, before we can proceed with the publication of your manuscript, please take a look at this suggestion and provide the requested analysis.

While going carefully through all the figures and source data I have also noticed that the source data for the EMD western blot do not seem to correspond to the western blot bands in the final figure 2C. The source data for FATE and Tom20 western blots do, however correspond to the final figure). Please check the data for this figure carefully and clarify this issue to me.

I trust that the final revision of your study would not take too much time, but please do let me know when you are planning to submit the final version.

REFEREE REPORTS

Referee #1:

I'm very pleased that the authors have resolved the issue about the localization of FATE1, and thankful that they made the effort to look into my suggestion. I think the story opens new avenues of research into the regulation of the MAM, and marks an important contribution to the field.

Referee #3:

While most of the concerns have been addressed in the revised version of the manuscript by Doghman-Bouguerra et al, it remains puzzling that FATE1 shows such a clear mitochondrial localization in the fluorescence microscopy experiments. In the revised version of the discussion, the authors discuss their findings more carefully. The mitochondrial localization though remains an unexplained and not-discussed paradox. This remains a major weakness of the paper that needs to be addressed prior to publication.

It seems a reasonable hypothesis that FATE1 inserts into the ER membrane with its TMD domain and interacts with mitochondrial proteins via its CC domain. Do the authors interpret this observation to indicate that the majority of mitochondrial tubules are lined by the ER? Or, do the authors hypothesize that FATE1 can interact with mitochondrial proteins in a conformation where the TMD is not inserted into the ER? The authors need to include a careful discussion of these possibilities in their manuscript.

The triple labeling presented in figure 2G is still of little value. For me it is impossible to tell,

whether the FATE1 signal localizes to mitochondria-ER interfaces. The insets provided in Figure S5 provide better insight into this question, however, Fig. S5 also gives the impression that FATE1 localizes to mitochondrial tubules also in areas where no ER crosses the mitochondria. Rather than determining fluorescent intensities along lines drawn randomly across the cell, it would be more interesting to trace the mitochondrial signal and determine the intensities of FATE1 on this trace and test whether there is an increase in FATE1 intensity in regions where ER tubules cross the mitochondria. If this is not the case, the finding should be discussed accordingly. Can FATE1 localize to mitochondria even if the ER is not in proximity (e. g. the TMD domain is not inserted into the ER).

Minor comment: p.7: "In addition, previous reports showed that a sizeable pool of extracellular EMD is localized in the ER", extracellular should be replaced by extranuclear.

3rd Revision - authors' response 10 June 2016

Reviewer #1
We are very glad that this Reviewer is satisfied with the modifications we made in the previous version of our manuscript and appreciated the novelty and the importance of our study.

Reviewer #3
We thank this Reviewer for his/her general appreciation of our work and for the interesting and constructive suggestions s/he gave to further reinforce our work. Following this Reviewer's indications, using a new in-house developed macro software we have now included data showing that FATE1 is significantly enriched in correspondence of ER-mitochondria contact sites in human adrenocortical cancer cells (Fig. 2G). In addition, we have discussed in detail the interesting suggestion given by this Reviewer that association of FATE1 with the mitochondrial surface at sites that are not in close contact with the ER may be mediated by association with mitochondrial partners in situations where the FATE1 C-terminal TMD may not be functional due to misfolding or shielding interactions with other partners (page 14).

We have also corrected the mistake at page 7 in the text indicated by the Reviewer.

4th Editorial Decision 16 June 2016

Thank you for your patience while I was waiting to hear back from referee 3 on the revised version of your study. I am happy to tell you that this reviewer is satisfied with the way in which you have addressed his/her concern about the mitochondrial localisation of FATE1 and I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Enzo LALLI
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41504-T

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	A series of 62 normal and benign steroidogenic tissues, 141 adrenocortical carcinomas and 77 non-steroidogenic malignancies were included in the study.
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	No, because it is easy for an experienced pathologist to assess the histological type and malignancy of a particular sample.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used non-parametric tests.
Is there an estimate of variation within each group of data?	Yes, variation is shown in each figure as SEM.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All primary antibodies used in this study are shown in Appendix Table S2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Our cell lines were recently authenticated by STR profiling and are regularly tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethics committee of the University of Würzburg (Germany).
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.	Informed consent was obtained from all subjects from whom tissue samples and clinical data were used for our study. All the experiments conformed to the principles set out in the Declaration of Helsinki and the HHS Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	We have followed REMARK reporting guidelines.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. 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.	No