EMBO reports

MIRO-1 interacts with VDAC-1 to regulate mitochondrial membrane potential in C. elegans

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Dear Dr. Ren,

Thank you for the submission of your research manuscript to EMBO reports. It has been seen by three experts in the field, and we have now received the full set of their reports (appended below).

As you will see, all referees acknowledge that certain aspects of this work are potentially interesting. However, they also identify limitations in the study and they mention that parts of the proposed model require further experimental support. In particular, we agree with referees #1 and #3 that more comprehensive biochemical characterization of the interaction between MIRO-1 and VDAC-1 is required to substantiate the conclusions of the study. In addition, we would like to emphasize the importance of the concern of referee #3 that the observed effects of the point mutation in MIRO-1 could be attributed to the impairment of its GTPase domain rather than to the abolishment of its interaction with VDAC-1; further experiments are required to distinguish between those two possibilities. Furthermore, referee #1 points out that VDAC-1 oligomerization has been linked to mitochondrial fragmentation, and investigation of the possibility that the interaction between MIRO-1 and VDAC-1 interferes with oligomerization of the latter would be advisable. Moreover, the referees provide a number of additional suggestions for the improvement of the work, which should be addressed. We note that although the broader analysis of the impact of the proposed interaction on mitochondrial function (as suggested by referee #3) would be desirable, it will not be required for further consideration of the manuscript.

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (February 16th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

Please note that you can publish the study either as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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

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7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the following datasets/data:
- Proteomic - mass spectrometry data

Please remember to provide a reviewer password if the datasets are not yet public.

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Data availability

The dataset produced in this study is available in the following database:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
*** Note - All links should resolve to a page where the data can be accessed. ***

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy (<https://www.embopress.org/competing-interests>) and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
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- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

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Yours sincerely,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The manuscript examines why some of the fragmented mitochondria can maintain sufficient function that will allow for survival of the C. elegance mutant. Their work suggests that Miro-1 may be interacting with VDAC-1 and thereby offering some functional protection to the fragmented mitochondria in this model.
Overall, all work presented here suggest an association between Miro-1 and protection by ATP maintenance. While this is an interesting observation, there are a few questions both technically and conceptually that need to be addressed.
A technical question: Why tubulin has been used as a loading control for mitochondria? This is not specific for mitochondria. Why not using another marker specific to mitochondria? Such as Hsp proteins, or VDAC1 and porin or COX?
Conceptual questions: The quality of the figures are low and hard to evaluate the expression pattern, however, are there any correlation between the size and MIRO-1 content? Can fragmented mitochondria be sorted based on their size to see if there are such correlation between size and normal functionality?
The need for further experiments: What is the mechanisms of action for Miro-1 and VDAC1 interaction? VDAC-1 oligomerization has been shown to be involved in mitochondrial fragmentation (PMID 30421242). This can be simply shown by cross-linking

experiments using EGS to capture the oligomeric states of VDAC1 by western blotting. This technique will allow if interaction of MIRO-1 and VDAC-1 can prevent VDAC1 oligomerization.
Also if calcium signaling is affected, can this be shown experimentally?

The word stochastic has been used several times throughout the text. It would be useful to better define what is meant by that? Randomly?

Referee #2:

In this manuscript, X. Ren et al studied the interaction between Miro-1 and VDAC-1, and proposed a membrane potential maintaining role specifically for fragment mitochondria, which would draw many attention. This is very interesting and novel study, and have good significance for the field.

1, Beside the overexpressed Miro1-GFP to demonstrate that more Miro-1 on fragment mitochondria (fig. 1D, E; Fig. 2A), the endogenous Miro1 is also suggested to be detected. Furthermore, the author nicely provided a wound model and show some accumulation of Miro1 on fragment mitochondria (fig 2A), did it also show a membrane potential correlation as fig 1A ?

2. The IP experiment: suggest an IP by VDAC and detect endogenous Miro-1. Also, in discussion, MIRO-1 interacting with VDAC-1: Does it mean that MIRO1 promoting VDAC activity, but covering its channel pore to maintain $\Delta\Psi m$? In addition, both the MIRO-1 and VDAC-1 are MOM proteins, but regulate $\Delta\Psi m$, which is keeping by the mitochondrial inner membrane? It needs more explanation in more detail.

Minor: In page 3, the second paragraph, "mitochondrial inner membrane space". May be "mitochondrial intermembrane space" is accurate.

Referee #3:

In this work, a connection between MIRO1, a rho GTPase involved in mitochondrial distrubution, and VDAC is proposed that directly determines maintenance of an inner membrane potential. The study shows that MIRO1 association with mitochondria occurs in C.elegans as in other systems, that this association is increased during wound healing and that MIRO1 function is required for normal mitochondrial fitness. The authors then provide some evidence that VDAC, the most abundant outer membrane protein could be a receptor for MIRO1 and that point mutations in MIRO1 can affect the interaction between both proteins. The authors then postulate that this interaction is critical to maintain the membrane potential over the inner membrane, but how this is mediated is largely unclear. While certain aspects of this work are potentially interesting, key parts of the proposed model lack experimental support or appear to be not work out well.

In order to improve the study for future submissions, the authors should more rigorously describe the proposed MIRO1-VDAC interaction biochemically (Visualization of a complex by additional experiments like BN-PAGE, gel filtration or chemical crosslinking including specificity controls). Moreover, as it stands now, the mechanism by which this potential complex impacts maintenance of the membrane potential is currently completely unclear and needs extensive new experimentation, ie are these effects of the point mutation in MIRO1 directly related the interaction with VDAC or are they secondary due to impairment of the GTPase domain in MIRO? Which aspects of mitochondrial physiology is impaired in these mutants, is the respiratory chain intact and assembled, can the complexes perform normal function, is there a problem with mtDNA expression, is the effect due to problems associated with metabolism of certain substrates, etc.

Response to Reviewers' comments (Our responses are in blue font):

Referee #1:

The manuscript examines why some of the fragmented mitochondria can maintain sufficient function that will allow for survival of the C. elegance mutant. Their work suggests that Miro-1 may be interacting with VDAC-1 and thereby offering some functional protection to the fragmented mitochondria in this model. Overall, all work presented here suggest an association between Miro-1 and protection by ATP maintenance. While this is an interesting observation, there are a few questions both technically and conceptually that need to be addressed.

RESPONSE: We thank the Reviewer for the positive comments.

A technical question: Why tubulin has been used as a loading control for mitochondria? This is not specific for mitochondria. Why not using another marker specific to mitochondria? Such as Hsp proteins, or VDAC1 and porin or COX?

RESPONSE: Thanks for the suggestions, this is a valid point. We agree with the Reviewer that it would be more precise to use mitochondrial protein as a loading control. To do this, we have now performed a new western blot by adding COX-4 as an internal control. Our new results showed that MIRO-1 is upregulated in *fzo-1* mutant as well as after wounding compared to COX-4 expression level. We have included this data in Fig 1C and Fig 2D.

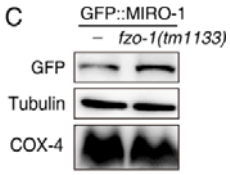


Fig 1C. Protein levels of MIRO-1 in *fzo-1* mutant. Immunoblot showing protein levels of MIRO-1 in WT and *fzo-1* mutant animals. Tubulin and COX-4 serve as loading controls.

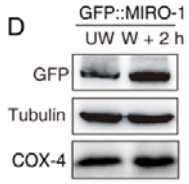


Fig 2D. Protein levels of MIRO-1 after wounding. Immunoblot showing protein levels of MIRO-1 before and after wounding. Tubulin and COX-4 serve as loading controls.

Conceptual questions: The quality of the figures are low and hard to evaluate the expression pattern, however, are there any correlation between the size and MIRO-1 content? Can fragmented mitochondria be sorted based on their size to see if there are such correlation between size and normal functionality?

RESPONSE: We apologize for the lower resolution of the figure that was prepared earlier due to compression. We have now improved the figure resolution by reducing the file compression.

To address whether there is any correlation between mitochondrial size and MIRO-1 content, we performed a new analysis using Spearman's rank correlation coefficient of fragmented mitochondria. Our results showed a significant but weak correlation between mitochondrial size and MIRO-1 content, suggesting that MIRO-1 tends to accumulate in larger mitochondria.

Additionally, we performed a new correlation analysis between fragmented mitochondria size and mitochondrial function, as indicated by the TMRE signal. Our new data demonstrated a strong correlation between TMRE signal and mitochondrial size, suggesting that larger mitochondria maintain the mitochondrial membrane potential to sustain function, which is consistent with the higher level of MIRO-1 localization in these mitochondria. Thanks for the excellent suggestions that have helped to improve our work. We have now included this data in Fig EV1E and Fig EV1F.

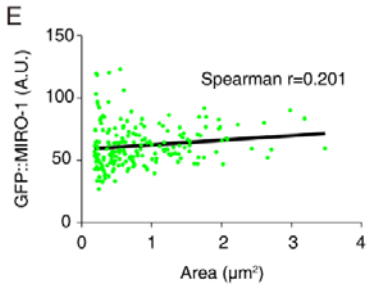


Fig EV1E. The correlation between the mitochondrial size and MIRO-1 content. Spearman's correlation analysis of the mitochondrial size and MIRO-1 expression in *fzo-1* mutant.

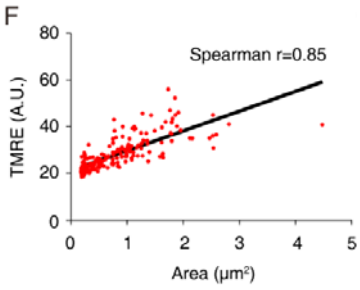


Fig EV1F. The correlation between mitochondrial size and mitochondrial membrane potential. Spearman's correlation analysis of the mitochondrial size and TMRE intensity in *fzo-1* mutant.

The need for further experiments: What is the mechanisms of action for Miro-1 and VDAC1 interaction? VDAC-1 oligomerization has been shown to be involved in mitochondrial fragmentation (PMID 30421242). This can be simply shown by crosslinking experiments using EGS to capture the oligomeric states of VDAC1 by western blotting. This technique will allow if interaction of MIRO-1 and VDAC-1 can prevent VDAC1 oligomerization.

RESPONSE: We thank the Reviewer for helpful comments and guidance in exploring the mechanism of the interaction between MIRO-1 and VDAC-1. To address this question, we conducted oligomerization experiments using crosslinking agents BMH (Bismaleimidoethane), which was commonly used in mitochondrial protein oligomerization (Prudent *et al*, 2015), to study VDAC-1 oligomerization. (We could not get EGS on time because of the shipping issue at the end of last year during the Covid-19 pandemic). Our new results showed that BMH treatment preserved VDAC-1 oligomerization in WT and miro-1 mutants. Interestingly, we observed that the oligomerization band of VDAC-1 in *miro-1(tm1966)* mutant was slightly stronger than that in WT animals. These results suggest that the loss of function of MIRO-1 results in an increased VDAC-1 oligomerization. Thus, it is likely that the interaction between MIRO-1 and VDAC-1 maintains the mitochondrial membrane potential by partially preventing VDAC-1 oligomerization. We have now included this data in the text (Fig 5H).

We would like to thank the Reviewer for insightful comments that helped us to mechanistically explain our findings.

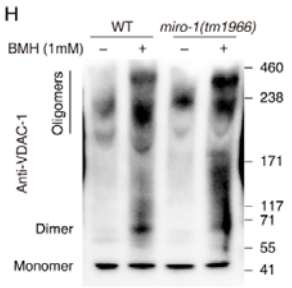
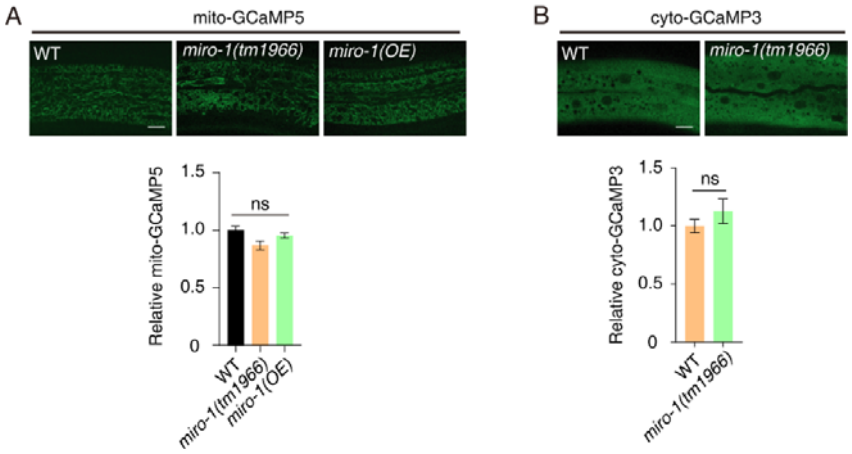


Fig 5H. Analysis of VDAC-1 oligomerization in WT and *miro-1(tm1966)* mutant by BMH. BN-PAGE and immunoblot analysis of VDAC-1 oligomerization crosslinked with BMH in WT and *miro-1(tm1966)* mutants using VDAC-1 antibody.

Also if calcium signaling is affected, can this be shown experimentally?

RESPONSE: To address if calcium is involved in MIRO-1 mediated regulation of mitochondrial membrane potential, we utilized transgenic *C. elegans* expressing mito-GCaMP5 and cyto-GCaMP3 to label mitochondrial and cytosolic calcium, respectively. By comparing the fluorescent signals of GCaMPs in the wild type and *miro-1(tm1966)* mutants, we did not observe significant differences in either mito-GCaMP5 and cyto-GCaMP3. These findings suggest that loss of

function of MIRO-1 does not significantly affect mitochondrial or cytosolic calcium levels.



Response Figure: Mitochondrial and cytosolic Ca^{2+} levels in WT, *miro-1(tm1966)*, *miro-1(OE).* (A) Fluorescence confocal microscopy images showing the expressions of mito::GCaMP5 in WT, *miro-1(tm1966)*, *miro-1(OE, overexpression)* mutants with statistical analysis. (B) Fluorescence confocal microscopy images showing the expressions of cyto::GCaMP3 in WT, *miro-1(tm1966)* mutants with statistical analysis.

The word stochastic has been used several times throughout the text. It would be useful to better define what is meant by that? Randomly?

RESPONSE: Thank you for your comments regarding using the term "stochastic" in our manuscript. We agree that this term can be ambiguous and appreciate the opportunity to provide further clarification. Our intention was to use "stochastic" to convey the concept of random events or fluctuations in mitochondrial dynamics, which is consistent with the widely accepted definition in the scientific literature (Chen *et al*, 2003). We have now revised our manuscript to provide additional explanation and context surrounding the use of this term. We thank the Reviewer for bringing this to our attention and believe that our revisions will better communicate the intended meaning of this term to our readers.

Referee #2:

In this manuscript, X. Ren et al studied the interaction between Miro-1 and VDAC-1, and proposed a membrane potential maintaining role specifically for fragment mitochondria, which would draw many attention. This is very interesting and novel study, and have good significance for the field.

RESPONSE: We thank the Reviewer for the positive comments on our findings.

Beside the overexpressed Miro1-GFP to demonstrate that more Miro-1 on fragment mitochondria (fig. 1D, E; Fig. 2A), the endogenous Miro1 is also suggested to be detected.

RESPONSE: We apologize for any confusion caused. In our study, we utilized *GFP::MIRO-1 (KI)* transgenic animals to examine the endogenous MIRO-1 expression. GFP::MIRO-1 images shown in Fig 1D and E, and Fig 2A (revised 2C) were captured from the knock-in strain, reflecting the endogenous MIRO-1 expression. We have now clarified this in the main text.

Furthermore, the author nicely provided a wound model and show some accumulation of Miro1 on fragment mitochondria (fig 2A), did it also show a membrane potential correlation as fig 1A ?

RESPONSE: To address the Reviewer's question, we performed a similar analysis to that presented in Fig 1A. Our findings revealed heterogeneity in the TMRE signal among fragmented mitochondria, with varying levels of TMRE signal observed, while a few mitochondria still maintained a strong TMRE signal.

Moreover, we found that the GFP::MIRO-1 signal was elevated in fragmented mitochondria with higher TMRE signals. Conversely, a barely detectable GFP::MIRO-1 signal in mitochondria with diminished TMRE signals. These results suggest that MIRO-1 accumulates on the fragmented mitochondria with elevated mitochondrial membrane potential. We have included this result in Fig 2A and B.

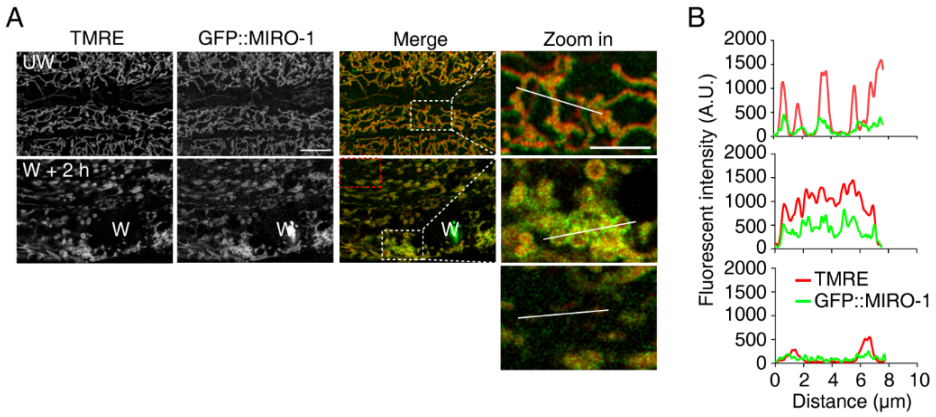


Fig 2A and B. The relationship of MIRO-1 expression and ΔΨm before and after wounding.

Fluorescence confocal microscopy images showing TMRE staining and GFP::MIRO-1 in WT animals with and without needle wounding.

2. The IP experiment: suggest an IP by VDAC and detect endogenous Miro-1.

RESPONSE: We performed a new co-IP experiment using VDAC-1 antibody-conjugated agarose beads to pulldown MIRO-1 in *GFP::MIRO-1(KI)* animals. Our results showed that GFP::MIRO-1 was detectable after pulldown by the VDAC-1 antibody, suggesting that MIRO-1 interacts with VDAC-1 at the endogenous level. We have now included this result in Fig 4E.

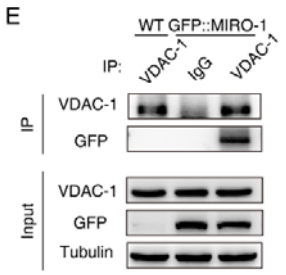


Fig 4E. VDAC-1 interacts with MIRO-1 *in vivo*. IP by VDAC-1 antibody and IB by GFP antibody.

Also, in discussion, MIRO-1 interacting with VDAC-1: Does it mean that MIRO1 promoting VDAC activity, but covering its channel pore to maintain ΔΨm?

RESPONSE: We thank the Reviewer for the insightful comments.

While we have shown an interaction between MIRO-1 and VDAC-1, we cannot definitively propose that MIRO-1 promotes VDAC-1 activity at this stage. We appreciate the insights provided by Reviewer 1, and in response, we have performed VDAC-1 oligomerization experiment. The result suggests that the absence of MIRO-1 could lead to an increased VDAC-1 oligomerization (Fig 5H). Therefore, we proposed that the interaction between MIRO-1 and VDAC-1 might regulate VDAC-1 channel activity through VDAC-1 oligomerization to maintain ΔΨm.

However, further experiments are necessary to confirm this hypothesis. Specifically, a patch-clamp investigation or the structure of the complex would be required to directly examine the VDAC-1 channel conductance in the presence and absence of MIRO-1. Once again, we appreciate the valuable feedback provided by the Reviewer and will take these suggestions into account in our ongoing research.

In addition, both the MIRO-1 and VDAC-1 are MOM proteins, but regulate ΔΨm, which is keeping by the mitochondrial inner membrane? It needs more explanation in more detail.

RESPONSE: We thank the Reviewer's insightful question, which we have pondered over for a long time. While it is true that the electrochemical gradient across the mitochondrial inner

membrane that underlies ΔΨm is primarily regulated by the electron transport chain (ETC) complexes, it is important to note that proteins located on the mitochondrial outer membrane can also impact ΔΨm. For example, Bcl-xL, a protein on the outer membrane, and even cytosolic proteins like Valosin-containing protein (VCP) have been reported to play a role in regulating ΔΨm (Bartolome *et al*, 2013; Vander Heiden *et al*, 1997).

As an outer mitochondrial membrane protein, MIRO-1 has only three amino acids located at the intermembrane space, making it unlikely that these directly interact with and regulate proteins on the inner membrane to maintain ΔΨm. VDAC-1 is known to play a critical role in ion transport, and there are numerous reports which have established a relationship between VDAC-1 and mitochondrial health as well as ΔΨm (Monaco *et al*, 2015; Zhou *et al*, 2019). Based on our current findings of the interaction between VDAC-1 and MIRO-1, we hypothesize that their interaction may significantly impact the ΔΨm through the regulation of the channel activity of VDAC-1. Further studies (e.g. the structure of this complex) are needed to elucidate the exact mechanism by which MIRO-1 and VDAC-1 interaction affects the ΔΨm.

Minor: In page 3, the second paragraph, "mitochondrial inner membrane space". May be "mitochondrial intermembrane space" is accurate.
RESPONSE: We have corrected the errors.

Referee #3:

In this work, a connection between MIRO1, a rho GTPase involved in mitochondrial distrubution, and VDAC is proposed that directly determines maintenance of an inner membrane potential. The study shows that MIRO1 association with mitochondria occurs in C.elegans as in other systems, that this association is increased during wound healing and that MIRO1 function is required for normal mitochondrial fitness. The authors then provide some evidence that VDAC, the most abundant outer membrane protein could be a receptor for MIRO1 and that point mutations in MIRO1 can affect the interaction between both proteins. The authors then postulate that this interaction is critical to maintain the membrane potential over the inner membrane, but how this is mediated is largely unclear. While certain aspects of this work are potentially interesting, key parts of the proposed model lack experimental support or appear to be not work out well.

RESPONSE: We thank the Reviewer for the positive comments and constructive insights that guided our revision.

In order to improve the study for future submissions, the authors should more rigorously describe the proposed MIRO1-VDAC interaction biochemically (Visualization of a complex by additional experiments like BN-PAGE, gel filtration or chemical crosslinking including specificity controls).

RESPONSE: We thank the Reviewer for the helpful suggestions. To address the Reviewer's concerns, we performed additional experiments to investigate the interaction between MIRO-1 and VDAC-1.

First, we collected total lysate proteins from *GFP::MIRO-1 (KI)* animals and treated them with BMH (Bismaleimidoethane) for crosslinking before subjecting them to BN-PAGE gel analysis. We found protein bands with similar molecular weight (MW) using both GFP and VDAC-1 antibodies, suggesting that MIRO-1 and VDAC-1 were present in the same complex. We have included this result in Fig 4F for clarity.

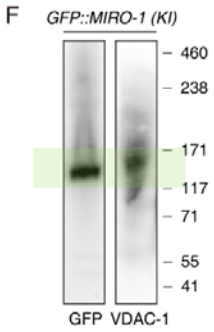


Fig 4F MIRO-1 formed a complex with VDAC-1 *in vivo.* BN-PAGE and immunoblot analysis of the total worm lysate of *GFP::MIRO-1 (KI)* by GFP and VDAC-1 antibody.

Moreover, we performed 2D SDS-PAGE analysis to examine if MIRO-1 could co-migrate with a fraction of VDAC-1. Our results revealed a small fraction of VDAC-1 can be detected in a high MW band in the first dimension BN-PAGE in *GFP::MIRO-1 (KI)*, suggesting MIRO-1 and VDAC-1 were in the same complex. In addition, we found this VDAC-1 signal in the 2nd dimension at the same MW was barely detectable in *GFP::MIRO-1 (E473G, KI)* mutant animals. These new findings suggest that VDAC-1 and MIRO-1 form a complex *in vivo*, and this interaction is dependent on the MIRO-1(E473). We have added the results in Fig 5D.

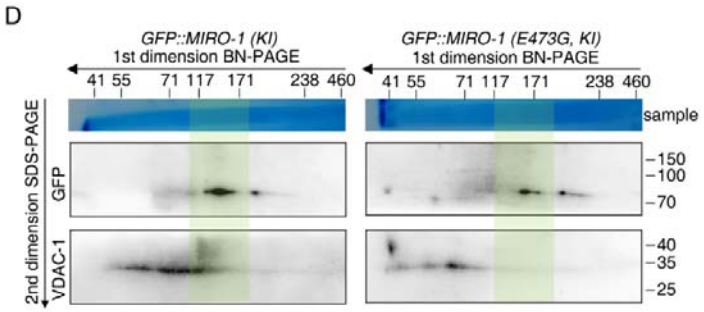


Fig 5D. MIRO-1 formed a complex with VDAC-1 *via* the residue E473 *in vivo.* First-dimension BN-PAGE and second-dimension SDS-PAGE analysis of total worm lysate of *GFP::MIRO-1 (KI)* and *GFP::MIRO-1 (E473G, KI)* by GFP and VDAC-1 antibody.

Furthermore, to investigate whether MIRO-1 and VDAC-1 form a complex and whether E473 in MIRO-1 is required, we purified the MIRO-1(dTM), MIRO-1(dTM, E473G), and VDAC-1 proteins, mixed them with the chemical crosslinker BMH. We then performed the SDS-PAGE gel to detect the proteins with anti-His antibody. We found a high molecular weight protein complex formed, indicating the interaction between MIRO-1 and VDAC-1 and forming a complex (lane 7). Notably, MIRO-1 (dTM, E473G) showed a reduced ability to form a protein complex with VDAC-1 (lane 6). These results support that MIRO-1 and VDAC-1 form a protein complex *in vitro*. We have now included this data in Fig 5E.

Together, our newly collected data suggests that MIRO-1 and VDAC-1 form a complex *in vivo*

and *in vitro*. Thanks to the Reviewer for the excellent suggestions that have helped improve our manuscript.

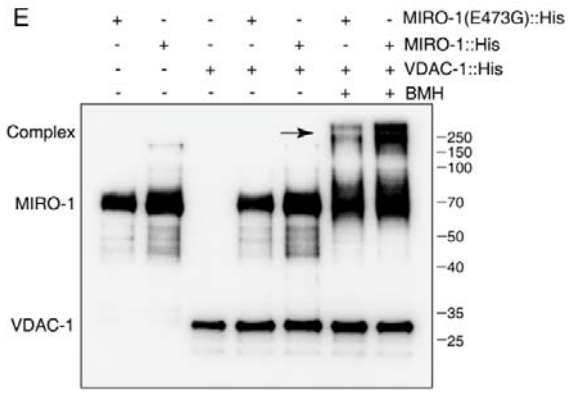


Fig 5E. MIRO-1 formed a complex with VDAC-1 *via* the residue E473 *in vitro*. Immunoblot analysis of purified MIRO-1 (dTM), MIRO-1 (dTM, E473G), and VDAC-1 proteins after BMH crosslinking by His antibody.

Moreover, as it stands now, the mechanism by which this potential complex impacts maintenance of the membrane potential is currently completely unclear and needs extensive new experimentation, ie are these effects of the point mutation in MIRO1 directly related the interaction with VDAC or are they secondary due to impairment of the GTPase domain in MIRO?

RESPONSE: We thank the Reviewer for the excellent suggestions. To address if the point mutation of E473G in MIRO-1 affects the GTPase activity, we performed a few experiments. First, we used GFP beads to pull down endogenous MIRO-1 from *GFP::MIRO-1 (KI)* and *GFP::MIRO-1(E473G, KI)* animals and then measured GTPase activity using a GTPase activity assay kit (Sigma, USA). We calculated the relative GTPase activity using protein concentration and GFP expression as an internal control. Interestingly, our results showed that MIRO-1 E473G point mutation exhibited higher GTPase activity than WT MIRO-1. To validate this result, we purified MIRO-1 and MIRO-1 (E473G) proteins without the transmembrane (TM) domain and found that the purified MIRO-1 (E473G) protein showed higher GTPase activity. We have added this result in Fig EV5F and G.

Based on these findings, we concluded that the E473G point mutation in MIRO-1 increases MIRO-1 GTPase activity. As observed in both *miro-1(E473G)* mutant and *miro-1* deletion mutant, this leads to a reduction in mitochondrial membrane potential. These results, combined with the biochemical data on the interaction of MIRO-1 and VDAC-1, suggest a decrease in the interaction

between MIRO-1 and VDAC-1, rather than the GTPase activity in MIRO-1(E473G), leads to a reduction in mitochondrial membrane potential. However, we acknowledge that investigating how E473G mutation leads to an increased GTPase activity would be an intriguing topic. We thank the Reviewer for the valuable suggestion, which helps to clarify our findings.

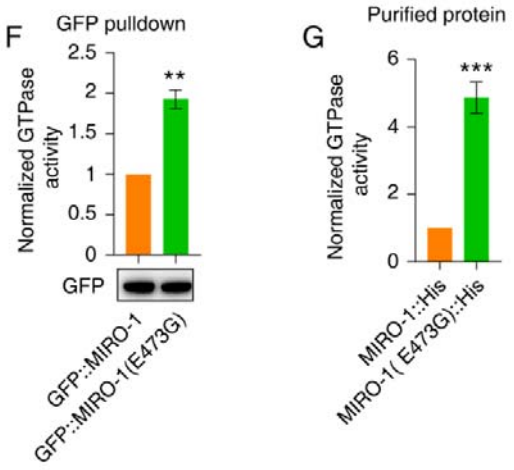


Fig EV5F and G. GTPase activity analysis of full-length MIRO-1 and E473G point mutation of MIRO-1.

Which aspects of mitochondrial physiology is impaired in these mutants, is the respiratory chain intact and assembled, can the complexes perform normal function, is there a problem with mtDNA expression, is the effect due to problems associated with metabolism of certain substrates, etc.

RESPONSE: We thank the Reviewer for bringing up the important question regarding the physiological effects of these mutants. Although our current study primarily focuses on the investigation of the molecular mechanism underlying the regulation of mitochondrial membrane potential through the interaction between MIRO-1 and VDAC-1, we acknowledge the significance of investigating the potential effects of these mutants on the other aspects of mitochondrial physiology. Accordingly, we will take this suggestion into account in our future research direction. Once again, we appreciate your insightful comments and suggestions.

References:

Bartolome F, Wu HC, Burchell VS, Preza E, Wray S, Mahoney CJ, Fox NC, Calvo A, Canosa A, Moglia C *et al* (2013) Pathogenic VCP mutations induce mitochondrial uncoupling and reduced ATP levels. *Neuron* 78: 57-64

Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* 160: 189-200

Monaco G, Decrock E, Arbel N, van Vliet AR, La Rovere RM, De Smedt H, Parys JB, Agostinis P,

Leybaert L, Shoshan-Barmatz V *et al* (2015) The BH4 domain of anti-apoptotic Bcl-XL, but not that of the related Bcl-2, limits the voltage-dependent anion channel 1 (VDAC1)-mediated transfer of pro-apoptotic Ca2+ signals to mitochondria. *J Biol Chem* 290: 9150-9161

Prudent J, Zunino R, Sugiura A, Mattie S, Shore GC, McBride HM (2015) MAPL SUMOylation of Drp1 Stabilizes an ER/Mitochondrial Platform Required for Cell Death. *Mol Cell* 59: 941-955

Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 91: 627-637

Zhou B, Kreuzer J, Kumsta C, Wu L, Kamer KJ, Cedillo L, Zhang Y, Li S, Kacergis MC, Webster CM *et al* (2019) Mitochondrial Permeability Uncouples Elevated Autophagy and Lifespan Extension. *Cell* 177: 299-314 e216

Dear Dr. Xu,

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of reports from the three referees that were asked to re-evaluate your study. Please find their comments included below.

As you will see, referees #1 and #2 mention that all of their previous concerns have been successfully addressed, and they now recommend publication. Referee #3 acknowledges that previously raised questions have been addressed during revision and new data have been included in the manuscript. However, referee #3 also points out that some of the conclusions are not fully supported by the new data, and some of the new experiments are not convincing or lack important controls. We note that further elucidation of the molecular mechanism by which MIRO-1 affects mitochondrial membrane potential is not required for further consideration of your work by EMBO reports.

Given these criticisms, we would like to invite you to revise your manuscript with the understanding that the concerns of referee #3 regarding the quality and conclusiveness of the added experiments and data (as detailed in their report) must be fully addressed and their suggestions taken on board. Please address all of their concerns in a complete point-by-point response, and make sure that all changes are highlighted in your revised manuscript to be clearly visible.

I would also like to note that there are several grammar mistakes throughout the manuscript, which we need you to correct. I recommend that the text be revised by a native English speaker or a language editing service.

From the editorial side, there are also a few things that we need from you:

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Editor
EMBO reports

Referee #1:

The authors have addressed all the points raised in my previous round of review. The key experiments are all supportive of their conclusions. I have no further comments and believe this can be published.

Referee #2:

The authors have answered my questions.

Referee #3:

In this revised manuscript, the authors have addresses a few questions raised on the initial version. Specifically, they now provide data on a putative complex between MIRO-1 and VDAC. This set of data is now part of the manuscript. The authors now have performed BN-PAGE analyses (Fig 4F), where they claim to observe a seemingly quantitative co-migration of the two proteins, as both proteins co-migrate at complexes of between 120-170 kDa. However, it is difficult to judge whether both proteins indeed co-migrate as the main fractions of MIRO-1 and VDAC are found at molecular weights that differ. Likewise, the authors performed 2D-SDS-BN-PAGE (Fig 5D) and claim that they observe a small fraction of MIRO-1 co-migrating with VDAC, which is lost in the MIRO-1 mutant. However, this experiment is far from convincing, as no specific complex formation can be observed and a longer exposure during the western blotting will most likely result in a similar signal in the MIRO-1 mutant as in the wild type. The authors added another experiment where they mix purified proteins in the test tube and observe a decreased chemical crosslinking of the proteins. This experiment looks convincing, but lacks control lanes where crosslinking was

performed on either MIRO-1 or VDAC alone. Hence, it is difficult to judge whether this very high molecular weight band (250 kDa) represents a specific complex that is then stabilized by the crosslinker, or whether this is an unspecific crosslinking of the purified proteins in the test tube, which would not be surprising given this experimental setup.
The authors also tested GTPase activity of purified WT and mutant versions of MIRO-1 and found that the mutant has increased activity. How this could influence the observed defect of the mutant on the mitochondrial membrane potential as well as the molecular mechanism by which MIRO-1 affects mitochondrial membrane potential remain elusive.

Response to Reviewers' comments (Our responses are in blue font):

Referee #1:

The authors have addressed all the points raised in my previous round of review. The key experiments are all supportive of their conclusions. I have no further comments and believe this can be published.

RESPONSE: We thank the Reviewer for accepting our revision.

Referee #2:

The authors have answered my questions.

RESPONSE: We thank the Reviewer for accepting our revision.

Referee #3:

In this revised manuscript, the authors have addresses a few questions raised on the initial version. Specifically, they now provide data on a putative complex between MIRO-1 and VDAC. This set of data is now part of the manuscript. The authors now have performed BN-PAGE analyses (Fig 4F), where they claim to observe a seemingly quantitative co-migration of the two proteins, as both proteins co-migrate at complexes of between 120-170 kDa. However, it is difficult to judge whether both proteins indeed co-migrate as the main fractions of MIRO-1 and VDAC are found at molecular weights that differ.

RESPONSE: Thanks again for the Reviewer's additional comments. To address the Reviewer's concerns about the co-migration of the endogenous MIRO-1 and VDAC-1, we repeated the BN-PAGE analyses. We found that partial fractions of MIRO-1 and VDAC are found at the same molecular weights, suggesting MIRO-1 could interact with VDAC-1. However, the main fractions of MIRO-1 and VDAC are still found at different molecular weights. We reason that the endogenous expression of MIRO-1 and VDAC-1 could vary. As MIRO-1 and VDAC-1 also interact with other proteins, which may also affect the co-migration of these two proteins.

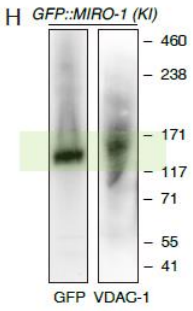

Fig 4H. MIRO-1 may form a complex with VDAC-1 *in vivo.* BN-PAGE and immunoblot analysis of the total worm lysate of *GFP::MIRO-1 (KI)* by GFP and VDAC-1 antibody.

Likewise, the authors performed 2D-SDS-BN-PAGE (Fig 5D) and claim that they observe a small fraction of MIRO-1 co-migrating with VDAC, which is lost in the MIRO-1 mutant. However, this experiment is far from convincing, as no specific complex formation can be observed and a longer exposure during the western blotting will most likely result in a similar signal in the MIRO-1 mutant as in the wild type.

RESPONSE: To address the Reviewer's concerns about the exposure issue on the western blot, we repeated the experiment simultaneously with the same exposure time. Consistent with our previous result, we found that the VDAC-1 signal in the 2nd dimension at the same MW was barely detectable in GFP::MIRO-1 (E473G, KI) mutants, compared with GFP::MIRO-1 (KI) animals. These results support our previous suggestion that VDAC-1 and MIRO-1 can interact with each other *in vivo*, and this interaction is dependent on the MIRO-1(E473). We have revised the results in Fig 6C.

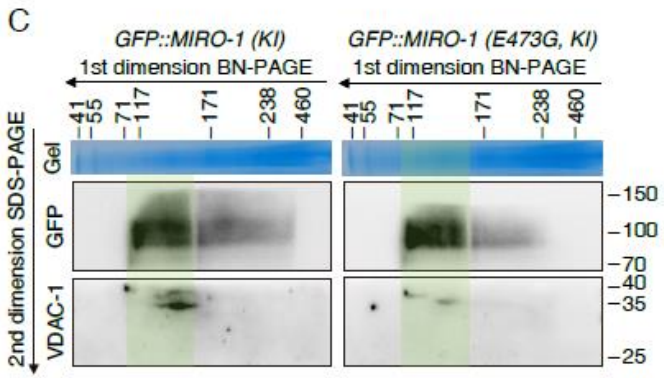

Fig 6C. MIRO-1 E473 is required for the interaction between MIRO-1 and VDAC-1. First-dimension BN-PAGE and second-dimension SDS-PAGE analysis of total worm lysate of *GFP::MIRO-1 (KI)* and *GFP::MIRO-1 (E473G, KI)* by GFP and VDAC-1 antibody.

The authors added another experiment where they mix purified proteins in the test tube and observe a decreased chemical crosslinking of the proteins. This experiment looks convincing, but lacks control lanes where crosslinking was performed on either MIRO-1 or VDAC alone. Hence, it is difficult to judge whether this very high molecular weight band (250 kDa) represents a specific

complex that is then stabilized by the crosslinker, or whether this is an unspecific crosslinking of the purified proteins in the test tube, which would not be surprising given this experimental setup.

RESPONSE: To address the Reviewer's concerns, we performed crosslinking assay again and analyzed the high molecular weight band (250 kDa) with mass spectrometry (MS), which identified both MIRO-1 and VDAC-1 within this band (Fig EV5D-F, and Table EV5). These findings suggest that MIRO-1 could interact with VDAC-1 and may form a complex *in vitro*. We have now included this newly collected data in the supplemental Figure.

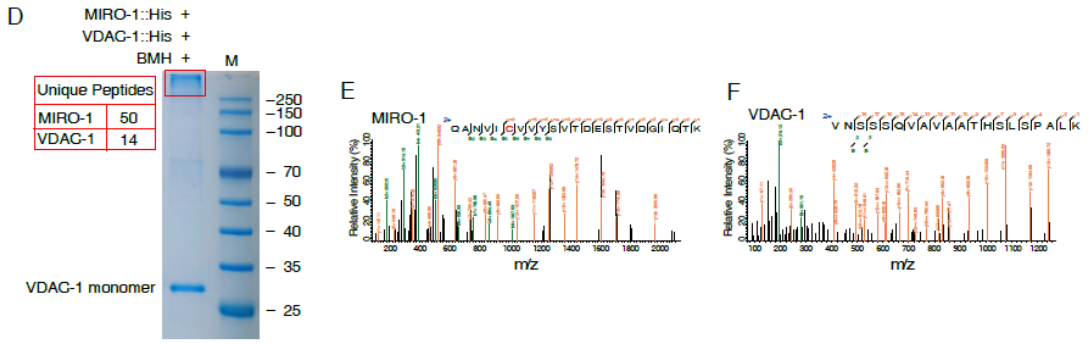

Fig EV5D-F. MIRO-1 interacts with VDAC-1 *in vitro*. A. Coomassie brilliant blue staining of purified MIRO-1 and VDAC-1 with BMH. B. Mass spectrometry analysis of high molecular weight gel from panel A.

Table EV5: MS identification of molecules in the high MW band.

Sample	Protein IDs	Peptide counts (unique)	Score	Coverage (%)
MIRO-1 and VDAC-1 with BMH	MIRO-1/VDAC-1	50/14	323.31/101.37	69.3/58

To further validate the interaction between VDAC-1 and MIRO-1, we performed an in vitro co-immunoprecipitation (co-IP) assay using purified fusion protein GFP::MIRO-1 and VDAC-1::His. The newly collected data supports that MIRO-1 interacts with VDAC-1 in vitro. We have now included this newly collected data in Fig 4F and G.

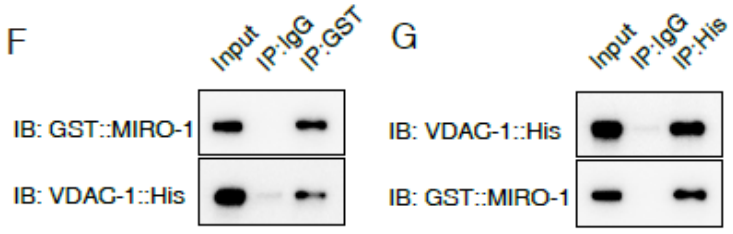

Fig 4F and G: Co-IP analysis of GST::MIRO-1 and VDAC-1::His fusion proteins.

The authors also tested GTPase activity of purified WT and mutant versions of MIRO-1 and found that the mutant has increased activity. How this could influence the observed defect of the mutant on the mitochondrial membrane potential as well as the molecular mechanism by which MIRO-1 affects mitochondrial membrane potential remain elusive.

RESPONSE: We are also puzzled about this interesting observation and believe this would be an intriguing topic for mechanistic study in the future. We thank the Reviewer for the valuable suggestion, which helps to clarify our findings.

Dr. Suhong Xu
Zhejiang University
School of Medicine
310058
China

Dear Dr. Xu,

Thank you for the submission of your revised manuscript for consideration by EMBO reports. We have now received the comments of referee #3, who explains that the new data now sufficiently support the conclusions of the study and recommends publication:

Referee 3:

"The authors have performed further experiments that strengthen their conclusions."

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

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- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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

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- 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.
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- 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.).
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  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods Section

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods Section

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods Section

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Not Applicable	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure section

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

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

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

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