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Intra-membrane client recognition potentiates the chaperone functions of Calnexin

Nicolas Bloemeke, Kevin Meighen-Berger, Manuel Hitzenberger, Nina Bach, Marina Parr, Joao Coelho, Dmitrij Frishman, Martin Zacharias, Stephan Sieber, and Matthias Feige
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Thank you for submitting your study on calnexin as membrane protein chaperone for our consideration. Two expert referees have now assessed it, with their reports copied below for your information. Since both reviewers acknowledge the interest of the findings and the general quality of the work, we shall be happy to consider a revised version further for publication in The EMBO Journal. The majority of the points raised relate to presentational/interpretational issues, but I would nevertheless ask you to diligently respond to all points at this stage, as it is our policy to consider only a single round of (major) revision.

Referee #1:

This manuscript presents data supporting the idea that calnexin (CNX) interacts with transmembrane proteins via its transmembrane segment and can do so independently of its lectin domain. The findings are interesting and convincing in general, and the manuscript is well-written, but there are some issues requiring clarification or justification. The results section begins with a mass spec (MS) analysis of proteins that were crosslinked to a tagged version of CNX. First, to the best of this reviewer's understanding, the experimental protocol would not rule out indirect interactions. The cross-linking was used only to capture proteins associated in some manner with CNX, and direct crosslinks were not identified. The results seem to be described and discussed without this caveat mentioned. Following the MS experiment, the authors introduce Cx32 to investigate the mechanism of interaction, though this protein seems to come from elsewhere rather than from the MS study. There is nothing wrong with that in principle, but the authors should be aware that the reader would have expected a validation of the MS results or some continuation based on the MS findings at that point in the text.

There are also a few issues of experimental design or interpretation. First, the conclusion on the top of page 8 that the binding is 6-fold "stronger" because the band on the western is that much more intense is not valid. Saying that the interaction is 6-fold stronger would require equilibrium affinity measurements, which were not done. The authors should stick to reporting the actual observation.

Another point relates to the Cx32 TMDs fused to the antibody constant region. In the legend to Fig. 2, the reader is told that "Sequences of TMD1 and TMD3 were inverted in the respective constructs to reflect the correct orientation within Cx32." This statement is horrifying to a protein biochemist. Peptides have polarity (distinct N and C termini), so stringing amino acids together in the opposite order does not "correct" anything, but rather generates a completely different peptide. It would be fair to say that the TMD1 and TMD3 sequences that appear in Fig. 2 have the same amino acid composition as two of the TMDs in Cx32, but they are not the same peptides in any sense! This fact must be disclosed in the main text. This issue seems to arise also with the sequence alignment used to design ConMem.

Talking about sequence alignments, one wonders whether the Tyr and Thr in the CNX TMD are conserved in CNX orthologs. It might make the case more convincing if they are. Regardless, the level of conservation should be mentioned.

The molecular dynamics simulations do not seem to be used as effectively as perhaps they could be. Is there anything to say about the relative orientations of the Tyr and Thr (and Leu) in the simulations? With what are they interacting?

The possibility of cation-pi interactions between Arg in interacting proteins and the Tyr in CNX would seem to exist but is not mentioned. Could simulations shed any light on this issue?

In Fig. 6B a "chase" rather than a "pulse-chase" experiment is shown. The data are quantified by normalizing the level of rhodopsin to its value when cycloheximide is added. However, it looks from the blot presented that there is already a lot less Rho at this point in the CNX Y/T/L mutant than in the WT CNX sample, which would make sense if Rho is constantly being degraded. Should the steady-state levels of Rho in both cases (or all three if empty vector is included) also be quantified and presented?

Very minor points:

There is no need to say "presumably inert Val residues." Just "Val residues" is enough, without presuming anything.

There is a typo near the top of page 11: "removes N-linked sugars not further modified further in the Golgi"

Figures:

The type on Fig. 1C is very small. Would it be possible to present the data on a non-linear scale such that the region in the lower left would be expanded and the protein name label size could be increased?

The black type on dark gray is a bit difficult to see in Fig. 1D. It might be better to lighten the gray TM helices. This issue is relevant to some of the schematics in other figures as well.

In Fig. 1E there are a number of bands that seem to be considered monomer or dimer. Perhaps a bracket rather than an arrow would clarify that this region of the gel, rather than one of the many bands, should be considered to represent the monomer (or dimer).

In Fig. 1G the GJ arrow doesn't really point to gap junctions as implied in the figure legend but rather to either a binucleated cell or to two attached cells.

The type in other figures, such as the helical wheel in Fig. 5A, is also very small. It would be helpful if the authors follow the EMBO press guidelines for figure labeling. Also, the Y, T, and L are colored slightly differently than the other amino acids in the helical wheel, but in my figure reproduction, this difference is very difficult to see.

Fig. 5B and 5C are not very useful, since the amino acids aren't labeled and it is not clear what the reader is supposed to understand from it. It is also not clear what the two side panels with the side chains in sticks are supposed to show, and nothing is said about them in the legend.

Referee #2:

Calnexin is well-known as a carbohydrate-dependent chaperone that binds to clients using its lectin domain that possess monoglucosylated glycans. It has also been proposed to have protein-protein interactions abilities for many years but the basis and validity of these interactions have been questioned. This study by Bloemeke et al., identifies and characterizes the basis for calnexin's protein-protein interaction with transmembrane proteins. They discover that this interaction involves membrane regions of the substrate and calnexin interacting using cleverly designed constructs for calnexin, natural substrates and a computationally designed substrate. They systematically dissect the interaction axis in the substrate and calnexin's TMD (transmembrane domain). Overall, this study provides a convincing story of (1) that calnexin interactions includes sites within the lipid bilayer and (2) discovers the basis for these interactions in the substrate and calnexin.

The experiments are well designed, carried out, controlled and interpreted. The manuscript is also well-written.

Issues to address:
Figure 2E: why are there multiple bands with FLAG that do not appear to correspond in mobility to the MYC bands if they are two labels on the same construct? Which of the FLAG bands are the miniCNX construct?

In figure 6 the binding to Rho is delineated for lectin compared to protein-protein intramembrane contributions. A construct with the lectin domain of calnexin mutated would further support this conclusion.

Minor points:

Figures 3B needs to designate what the -/+ in the figure refers to.

Figure 5D cartoon should show tags for ease of following the experiment.

Pg 4 use of tether is misleading. It sounds more like a lipid or GPI anchor than a fully integrated TMD.

arguin on page 13 should be fixed.

Dear reviewers,

We would like to thank you very much for the positive evaluation as well as the constructive criticism on our study. Please find below a point-by-point reply where we have addressed your concerns by additional experiments as well as by editing the text and figures of our manuscript. We hope that our reply addresses all your concerns adequately and we would like to thank you again for the time you have invested in reviewing our manuscript.

Reviewer 1:

This manuscript presents data supporting the idea that calnexin (CNX) interacts with transmembrane proteins via its transmembrane segment and can do so independently of its lectin domain. The findings are interesting and convincing in general, and the manuscript is well-written, but there are some issues requiring clarification or justification. The results section begins with a mass spec (MS) analysis of proteins that were crosslinked to a tagged version of CNX. First, to the best of this reviewer's understanding, the experimental protocol would not rule out indirect interactions. The cross-linking was used only to capture proteins associated in some manner with CNX, and direct crosslinks were not identified. The results seem to be described and discussed without this caveat mentioned. Following the MS experiment, the authors introduce Cx32 to investigate the mechanism of interaction, though this protein seems to come from elsewhere rather than from the MS study. There is nothing wrong with that in principle, but the authors should be aware that the reader would have expected a validation of the MS results or some continuation based on the MS findings at that point in the text.

We agree with the argument that our approach could also identify indirect interactors. For this reason, we had included this point in the discussion of our submitted manuscript. In the revised manuscript we now also mention this in the corresponding results section. The same applies to the use of Cx32 as a model, where we now also give more explanation on our choice. We would also like to mention that we verified two of our MS hits by co-IP experiments in Figure S5C, when the intramembrane mutant was introduced in the study.

In addition to these points, since direct versus indirect interaction also intrigued us, we now include a new experiment in Figure 2C. CNX possesses two cysteines on the cytoplasmic side on its TM region (see Figure S2). We reasoned that these could possibly crosslink to substrate-intrinsic cysteines in proximity if the interaction was direct. We thus included a single Cys close to the cytoplasm within the Cx32 TMD1 that we found to co-IP with CNX (Figure 2B). Using BMH as a membrane-permeable crosslinker, we could observe crosslinks between CNX and Cx32 TMD1 (new Figure 2C), further supporting the idea of direct interactions between the CNX TMD and substrate TMDs.

There are also a few issues of experimental design or interpretation. First, the conclusion on the top of page 8 that the binding is 6-fold "stronger" because the band

on the western is that much more intense is not valid. Saying that the interaction is 6-fold stronger would require equilibrium affinity measurements, which were not done. The authors should stick to reporting the actual observation.

Following this suggestion, we have reworded this paragraph. We now mention a 6-fold stronger signal in the blots, arguing for stronger binding.

Another point relates to the Cx32 TMDs fused to the antibody constant region. In the legend to Fig. 2, the reader is told that "Sequences of TMD1 and TMD3 were inverted in the respective constructs to reflect the correct orientation within Cx32." This statement is horrifying to a protein biochemist. Peptides have polarity (distinct N and C termini), so stringing amino acids together in the opposite order does not "correct" anything, but rather generates a completely different peptide. It would be fair to say that the TMD1 and TMD3 sequences that appear in Fig. 2 have the same amino acid composition as two of the TMDs in Cx32, but they are not the same peptides in any sense! This fact must be disclosed in the main text. This issue seems to arise also with the sequence alignment used to design ConMem.

We excuse our negligence in this regard - of course the polarity matters. We thus now write that we inverted the sequence to have the same amino acid composition and the same amino acids exposed to the lipid bilayer – in the legend to Figure 2A and to Figure 3A.

Talking about sequence alignments, one wonders whether the Tyr and Thr in the CNX TMD are conserved in CNX orthologs. It might make the case more convincing if they are. Regardless, the level of conservation should be mentioned.

Picking up this idea, we now include an alignment of CNX TM domains from different species in SI Figure S7A. The entire TM domain, including the YxxT motif, of CNX is highly conserved.

The molecular dynamics simulations do not seem to be used as effectively as perhaps they could be. Is there anything to say about the relative orientations of the Tyr and Thr (and Leu) in the simulations? With what are they interacting? The possibility of cation-pi interactions between Arg in interacting proteins and the Tyr in CNX would seem to exist but is not mentioned. Could simulations shed any light on this issue?

We now extend the analyses our simulations in several ways. The Tyr and Thr were in the simulations always pointing towards the substrate helices, when a complex was observed. This is now mentioned in the results. Furthermore, we now mention more details on the kind of interactions we observed. These differ to a certain extent, but for Cx32-TMD1 and CNX, we indeed see polar interactions of the Tyr with the Cx32 backbone and possible Arg:Phe cation-pi interactions. This is now shown in the updated Figure 5C and described in the results. And lastly, we now include

simulation data on a CNX TMD mutant where YTL were replaced by Val (like in the experiments) leading to destabilized interaction with the ConMem TMD (again, like in the experiments).

In Fig. 6B a "chase" rather than a "pulse-chase" experiment is shown. The data are quantified by normalizing the level of rhodopsin to its value when cycloheximide is added. However, it looks from the blot presented that there is already a lot less Rho at this point in the CNX Y/T/L mutant than in the WT CNX sample, which would make sense if Rho is constantly being degraded. Should the steady-state levels of Rho in both cases (or all three if empty vector is included) also be quantified and presented?

We completely agree with this observation: the shortened half-lifes are fully consistent with reduced steady state levels, which we mention in the results. We consider the half-life analyses and this mentioning sufficient and hope the reviewer agrees. Of course this is a chase experiment and the text is now corrected.

Very minor points:

There is no need to say "presumably inert Val residues." Just "Val residues" is enough, without presuming anything.

Agreed and corrected.

There is a typo near the top of page 11: "removes N-linked sugars not further modified further in the Golgi"

Thank you for spotting this: corrected.

Figures:

The type on Fig. 1C is very small. Would it be possible to present the data on a non-linear scale such that the region in the lower left would be expanded and the protein name label size could be increased?

We now increased all font sizes.

The black type on dark gray is a bit difficult to see in Fig. 1D. It might be better to lighten the gray TM helices. This issue is relevant to some of the schematics in other figures as well.

Thank you for this comment, we now show lighter TM helices throughout.

In Fig. 1E there are a number of bands that seem to be considered monomer or dimer. Perhaps a bracket rather than an arrow would clarify that this region of the gel,

rather than one of the many bands, should be considered to represent the monomer (or dimer).

Thank you for this comment, we now show brackets for all figures where needed.

In Fig. 1G the GJ arrow doesn't really point to gap junctions as implied in the figure legend but rather to either a binucleated cell or to two attached cells.

We are not entirely sure where this comment aims at: the fluorescent plaques at the cell boundary correspond to the gap junctions. At those, the arrow points.

The type in other figures, such as the helical wheel in Fig. 5A, is also very small. It would be helpful if the authors follow the EMBO press guidelines for figure labeling. Also, the Y, T, and L are colored slightly differently than the other amino acids in the helical wheel, but in my figure reproduction, this difference is very difficult to see.

We now carefully checked all font sizes again and adjusted them were needed.

Fig. 5B and 5C are not very useful, since the amino acids aren't labeled and it is not clear what the reader is supposed to understand from it. It is also not clear what the two side panels with the side chains in sticks are supposed to show, and nothing is said about them in the legend.

We now significantly simplified Figure 5B and C, focusing on the residues relevant for interactions.

Reviewer 2:

Calnexin is well-known as a carbohydrate-dependent chaperone that binds to clients using its lectin domain that possess monoglucosylated glycans. It has also been proposed to have protein-protein interactions abilities for many years but the basis and validity of these interactions have been questioned. This study by Bloemeke et al., identifies and characterizes the basis for calnexin's protein-protein interaction with transmembrane proteins. They discover that this interaction involves membrane regions of the substrate and calnexin interacting using cleverly designed constructs for calnexin, natural substrates and a computationally designed substrate. They systematically dissect the interaction axis in the substrate and calnexin's TMD (transmembrane domain). Overall, this study provides a convincing story of (1) that calnexin interactions includes sites within the lipid bilayer and (2) discovers the basis for these interactions in the substrate and calnexin.

The experiments are well designed, carried out, controlled and interpreted. The manuscript is also well-written.

Issues to address:
Figure 2E: why are there multiple bands with FLAG that do not appear to correspond in mobility to the MYC bands if they are two labels on the same construct? Which of the FLAG bands are the miniCNX construct?

We have repeated and extended this experiment now using different IP/immunoblotting antibodies. This solved the problem and the new data can be found in Appendix Fig S2E.

In figure 6 the binding to Rho is delineated for lectin compared to protein-protein intramembrane contributions. A construct with the lectin domain of calnexin mutated would further support this conclusion.

This is a very good idea, which we happily picked up. We individually tested two different lectin mutants of CNX described in the literature (Y164A and E216A, Schrag et al, 2001, Mol Cell). However, when we expressed these in CNX k/o cells, we could not detect any monomeric Rhodopsin any more, only low levels of high molecular weight species/aggregates. Whereas this supports an important role of the lectin domain in Rho stabilization, it unfortunately precluded any further analyses.

Minor points:
Figures 3B needs to designate what the -/+ in the figure refers to.

The -/+ refers to the absence/presence of either EndoH or PNGaseF; to make this clearer, we have now changed the labeling.

Figure 5D cartoon should show tags for ease of following the experiment.

Following this suggestion, we now added the tags to the cartoons.

Pg 4 use of tether is misleading. It sounds more like a lipid or GPI anchor than a fully integrated TMD.

We changed the wording to “integrated into”.

arguin on page 13 should be fixed.
Thank you for spotting this, has been fixed.

Thank you for submitting your revised manuscript for our consideration. With some delay caused by my absence from the office, I have now had it re-reviewed by the original referee 1, whose comments are copied below for your information. As you will see, the referee is largely satisfied with your revisions, but retains a few specific concerns, which I would kindly ask you to incorporate as appropriate during a final round of minor revision.

Referee #1:

The authors have done a serious and thorough job of considering the reviewers' questions and suggestions. For the most part, the manuscript makes a convincing case for the authors' main points. I will just raise one last issue that the authors may want to consider before finalizing their manuscript:

This reviewer does not fully understand the new results using the BMH cross-linker. Though it is not labeled on the gel, the band around 250 kDa in the WT crosslinked lane of the input is presumably CNX dimer. What is not clear is why the putatively crosslinked immunoprecipitated product seems to migrate at the same position as CNX alone. A ~15 kDa shift should in principle be detectable. To convince the reader (and perhaps the authors themselves) that the observed species is really what is claimed, a lane containing a comparable small amount of CNX control should be run on the same gel to determine whether there is indeed a shift.

Small things:

Here is the origin of the confusion regarding the gap junctions. When the reader (or at least this reader) looks/looked at Figure 1F (sorry, it wasn't Figure 1G as I had written in my previous review), it is/was not clear that these cells are confluent. Only when one zooms in a lot on the figure (which I did not do the first time) can one see that faint PDI labeling extends from the left cell all the way to the cell that is pointed out with the GJ label. The fact that the GJ label points to a region of the central cell (or cells) that is not really facing another cell made me think that the red was just cell-surface connexin rather than gap junctions. Also, since there is only one arrow, but presumably many gap junctions, it looks like the arrow is just pointing out the central cell pair and not anything specific on it. What might be helpful is an inset showing a zoom-in on the cell-cell junction, or just putting a few, thinner, arrows from the "GJ" label to a few regions at the junction between the central and left cell. Also, the figure legend could clarify that the gap junctions are indeed at the interface between cells by saying something like: "GJ denotes gap junctions observed at cell-cell junctions in cultures transfected with WT Cx32."

p. 7, it should be "a common genetic disorder"

p. 9, what is meant by "against" in "we replaced an Arg at a suitable position against Cys"? Is the intention that the Arg was mutated to Cys? If so, "replaced... with" rather than "replaced... against" may be clearer. Or just "We mutated an Arg at a suitable position to Cys."

The same situation arises on p. 14: "when the YTL-motif was exchanged against Ala instead of Val." More natural would be: "when the YTL motif was replaced by alanines instead of valines." It is generally clearer, though to write Y/T/L was mutated to V/V/V or A/A/A.

Fig. 2 legend: "sequences is mis-spelled in "fused to seuences of interest"

We thank the reviewer for the positive feedback on our revised work, and for the very thorough and constructive review process. In the following, please find a reply to the remaining points raised.

Referee #1:

The authors have done a serious and thorough job of considering the reviewers' questions and suggestions. For the most part, the manuscript makes a convincing case for the authors' main points. I will just raise one last issue that the authors may want to consider before finalizing their manuscript:

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Although we did not test this further, we also consider the band around 250 kDa to be CNX dimers. The running behavior indeed is a valid observation. We always run two molecular weight markers on our blots, one on each side, so that we could very carefully again compare the running behavior of CNX alone with the x-linked species. Only a very small upward shift is observable so that the x-link apparently does not lead to any major change in CNX migration. This may e.g. be due to different SDS binding of the adduct or its rather compact size due to x-linking. Since we have two specificity-conferring steps (IP against CL and blot against V5) and, as a further control, the x-link depends on the two cysteines at the C-terminal end of the CNX TMD we are confident that we have a correct x-linked species. Also, it should be noted that CNX itself always migrates at a larger molecular weight than expected (slightly above 100 kDa although its calculated MW is only around 70 kDa), which may be due to its very acidic pI of 4.5 and thus less SDS-binding; x-linking may lead to more “normal” SDS binding. We hope the reviewer can agree to this reasoning.

Small things:

Here is the origin of the confusion regarding the gap junctions. When the reader (or at least this reader) looks/looked at Figure 1F (sorry, it wasn't Figure 1G as I had written in my previous review), it is/was not clear that these cells are confluent. Only when one zooms in a lot on the figure (which I did not do the first time) can one see that faint PDI labeling extends from the left cell all the way to the cell that is pointed out with the GJ label. The fact that the GJ label points to a region of the central cell (or cells) that is not really facing another cell made me think that the red was just cell-

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We now, as suggested, show several arrows and excuse for this misleading labeling. Also, we happily took up the suggestion and edited the text of the legend.

p. 7, it should be "a common genetic disorder"

Thank you, corrected.

p. 9, what is meant by "against" in "we replaced an Arg at a suitable position against Cys"? Is the intention that the Arg was mutated to Cys? If so, "replaced... with" rather than "replaced... against" may be clearer. Or just "We mutated an Arg at a suitable position to Cys."

Thank you, changed to “with” as suggested. Also, we extended the text to make our idea clearer:

> *To assess if binding was direct, we made use of cysteine crosslinking in the membrane. CNX possesses two intrinsic Cys residues at the cytoplasmic side of its TMD (Appendix Fig S2D). We reasoned that these should be in proximity to the cytoplasmic side of a substrate TMD if interactions were direct. Thus, within the first TMD of Cx32, we replaced an Arg at a suitable position with Cys (Fig 2A).*

The same situation arises on p. 14: "when the YTL-motif was exchanged against Ala instead of Val." More natural would be: "when the YTL motif was replaced by alanines instead of valines." It is generally clearer, though to write Y/T/L was mutated to V/V/V or A/A/A.

Thank you, we changed the wording.

Fig. 2 legend: "sequences is mis-spelled in "fused to seuences of interest"

Thank you, corrected.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Prof. Dr. Matthias J. Feige
Journal Submitted to: Embo J
Manuscript Number: EMBOJ-2022-110959R

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Reporting Checklist for Life Science Articles (updated January
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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- 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

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 $\chi 2$ 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods and Figures

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Meterial and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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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	
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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	

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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.	Not Applicable	

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.	Yes	Material and Methods and Figures
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	Material and Methods and Figures

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.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Material and Material and Figures

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	
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Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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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	Supplementary SI tables (MS Data)
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?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	