THE EMBO JOURNAL

REDUCED ER-MITOCHONDRIA CONNECTIVITY PROMOTES NEUROBLASTOMA MULTIDRUG RESISTANCE

Jorida Coku, David Booth, Jan Skoda, Madison Pedrotty, Jennifer Vogel, Kangning Liu, Annette Vu, Erica Carpenter, Jamie Ye, Michelle Chen, Peter Dunbar, Elizabeth Scadden, Taekyung Yun, Eiko Nakamaru-Ogiso, Estela Area-Gomez, Yimei Li, Kelly Goldsmith, Patrick Reynolds, Gyorgy Hajnoczky, and Michael Hogarty
DOI: 10.15252/embj.2021108272

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Review Timeline:

Submission Date:	16th Mar 21
Editorial Decision:	3rd May 21
Revision Received:	24th Nov 21
Editorial Decision:	19th Dec 21
Revision Received:	14th Jan 22
Accepted:	24th Jan 22

Editor: Elisabetta Argenzio / Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **3rd May 2021**

Dear Dr. Hogarty,

Thank you for submitting your manuscript entitled "Reduced ER-mitochondria connectivity promotes neuroblastoma multidrug resistance" [EMBOJ-2021-108272] to The EMBO Journal. I have now read your letter and discussed it with the other members of our editorial team.

The consensus is that the plan to address referees' points seems to be reasonable. Given the overall interest of your study, I am pleased to invite submission of a revised manuscript as indicated in the reviewers' reports. I would like to point out that addressing all their points in a conclusive manner will be essential for publication in The EMBO Journal, as well as a strong support from the referees. Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process.

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 http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password if the datasets are not yet public.

We usually expect to receive revised manuscripts within three months of the first decision. We are aware that many laboratories cannot function at full pace during the current COVID-19 pandemic and thus can relax this deadline. Also, we can extend our 'scooping protection policy' to cover the period required for a full revision to address all of the experimental issues highlighted in the editorial decision letter. Please inform us as soon as a paper with related content would be published elsewhere.

Thank you again for the opportunity to consider this work for publication, and please feel free to contact me with any questions about submission of the revised manuscript to The EMBO Journal. I look forward to your revision.

Best regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 1st Aug 2021.

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Referee #1:

In this manuscript, Coku et al. examine the role of ER-mitochondria contacts (ERMCs) in mediating cancer therapy resistance. They isolated mitochondria from neuroblastoma cell lines obtained from the same patient at the time of diagnosis (DX) and at relapse following treatment (REL). They observed that mitochondria from REL had markedly attenuated apoptotic responses and are resistant to diverse cancer therapeutics compared to patient-matched DX. Moreover, they attributed these attenuated mitochondrial apoptotic responses to a reduction in the proportion of ERMCs. The observed phenotype is not reverted by the modulator of Ca2+ communications between the two organelles but is (partially) revered by KD the component of the ERMCs, mitofusins 2 and PACS2. They conclude that ER-mitochondria contact remodeling is required to acquire multidrug resistance but in a Ca2+ independent fashion.
While the study is of potential interest, I have a number of concerns that undermine confidence in these conclusions. There are significant methodological concerns, as well as a lack of a convincing mechanism for how alteration in ERMCs could contribute to therapy resistance. I cannot, therefore, positively recommend this manuscript for publication on EMBO. I will list all the major concerns, hoping to stimulate the author to improve their investigation.

- The starting experiments measure mitochondrial sensitivity from different patient cell lines to release cyt C when stimulated with tBid or BimBH3 and reported that most lines REL developed resistance to undergoes cyt C release. Authors utilize heavy membrane fraction (which contains mitochondria, MAMs, and other contaminants). In these conditions, this referee expects that ERMCs are not influential for MOMP execution, tBid and BimBH3 should be able to engage BAK regardless of the presence of ER. In this perspective, involving ERMCs investigation is not the most logical to explain the phenotype of Figure 1, and the lack of a role for Ca2+ transfer could be a confirmation of a lock of role for ERMCs.
If authors want to focus on a role for tBid and BimBH3 in the induction of cyt C release in isolated mitos, then a robust molecular background should be offered.
Also, the choice of mfn2 and PACS2 does not guarantee the manipulation of ERMCs only. Indeed MFN2 KD is reported to modify mitochondrial morphology, which could impact apoptosis sensitivity, and PACS2 is reported to relocate to mitochondria during stress conditions to favor MOMP.
- A second critical point is that the study is merely descriptive and lacks a detailed molecular mechanism. It is obscure how therapy-resistant cancer cells could display lower ER-mitochondria contacts. Not even just expression levels of the ERMCs components are offered.
- Authors stated that "reduced transfer of Ca2+ from ER to mitochondria is not required for mitochondrial desensitization towards apoptotic MOMP". They perform mitochondrial calcium measurement in two different DX/REL pairs obtaining opposite results; it is not clear how they explain these data. In front of the strong reduction in ERMCs described in Figure 3, a strong alteration in Ca2+ transfer is just expected. How do authors explain this observation? Coupling time is not the most used readout to verify this phenotype, while the elevation of [Ca2+]m is the most expected but not observed. Again, how could authors explain this

phenotype? Also, augmentation of ERMCs should favor cyt C release regardless of Ca2+, but this measurement was not reported.
- Authors stated that "Immunomagnetic-bead separation (30) induced less membrane disruption, and MOMP sensitivity in response to BimBH3 was greater for CHLA15 heavy membrane fractions (mitochondria with intact MAMs) compared with purified mitochondria (reduced MAMs; Figure 5A). They should demonstrate the different MAMs amount after this separation.

Referee #3:

In this study Coku and colleagues investigate the basis for multidrug resistance in neuroblastoma. Using matched lines, pre/post treatment (sensitive and resistant) they use BH3-profiling to demonstrate a defect in mitochondrial apoptosis, move into cell based assays. Investigating the mechanistic basis for this they propose defective mito-ER contacts (MAMs) as the basis for defective apoptosis induction hence drug-resistance. Various earlier studies have made links between MAMs and apoptotic sensitivity, prominently by Ca transfer (tested here) or sphingolipid metabolism. The main novelty in this study, in my opinion, centers on the proposal that MAMs are important for drug-sensitivity in neuroblastoma and that loss of MAMs can be selected for as a means of drug resistance. Nevertheless, I consider a definitive causative role for loss MAMs in drug resistance needs further validation as does why loss may contribute to drug resistance - mechanistically this remains unclear. These points, and others that should be addressed, are detailed below.

In my view further demonstration of the importance of loss of MAMs in the effects described ins required (Figure 5) - the limited proteolysis expt. (disrupting MAMs) is very difficult to control due to possible cleavage of proteins involved in MOMP (e.g BAK) leading indirectly to inhibitory effects on MOMP, PACS2 and MFN2 knockdown can of course have a lot of indirect effects, since both proteins have various purported functions. One prediction of the model would be that direct tethering of the ER to mitochondria would bypass apoptotic resistance in the relapse cells - this can be directly addressed using the excellent tools co-author Hajnockzy has previously made (PMID 20603080)

Addition to these expts, the effects of modulating MAM numbers on therapy induced death (agents used in Fig 2) should also be addressed, to determine the relevance of MAM in clinically relevant neuroblastoma therapies.

How MAMs contribute to apoptotic sensitivity here is not clear, the authors extensively investigated a role for Ca transfer (finding none), one other possibly based on the work by Chipuk and colleagues (discussed at various points) is through effects on sphingolipid generation/mitochondrial transfer supporting pro-apoptotic BAX activation, this should be investigated.

An obvious point of apoptotic deregulation is through upregulation of anti-apoptotic BCL-2 family members, the authors have previously discounted this in another study, this should also be highlighted where they state "loss of BAX or BAK was unlikely...."

Referee #4:

Interesting paper linking therapy resistance in relapsed patients to reduced endoplasmic reticulum-mitochondrial contacts. Quality of science is good, but all studies done in vitro and sample sizes are quite small. The authors did show that exposure of cells in vitro to ABT-737 led to acquired resistance. Do these cells acquire reduced MAMs? Can any conclusios about reduced ERMCs in response to therapy be associated with genetic aberrations in the neuroblastoma tumors?

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November 24, 2021

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Dear Dr. Klimmeck:

Thank you for the opportunity to resubmit our manuscript, *"Reduced ER-mitochondria connectivity promotes neuroblastoma multidrug resistance",* which we have extensively revised in response to the referee critiques. We respectfully submit this revision for consideration of publication as a Research Article.

The referee critiques were extremely constructive and useful. Overall, the reviewers found the work of great potential interest and impact but the lack of a more detailed mechanistic understanding of how reduced endoplasmic reticulum-mitochondria contacts (ERMCs) contribute to therapy resistance in cancer cells dampened enthusiasm. We address this in this revised manuscript, having identified a critical lipid transfer function attributable to ERMCs that regulates Bak and Bax sensitivity to undergo stress signal-induced oligomerization and induce mitochondrial outer membrane permeabilization (MOMP). The activity implicated is the synthesis and transfer of ceramide to mitochondria that occurs at functional ERMCs, as ceramide and its metabolites are required to support Bak and Bax oligomerization, and ceramide itself participates in outer mitochondrial membrane pore formation. We corroborate these alterations in lipid composition in relapsed cells with reduced ERMCs by showing reduced ceramides and elevated sphingomyelin:ceramide ratios using lipidomic analyses. We also now phenocopy resistance in otherwise stress-sensitive cells by biochemically blocking sphingomyelin to ceramide conversion. Collectively, our work identifies a novel organelle-contact mediated mechanism of multidrug cancer therapy resistance that has broad implications within cancer biology and provides opportunities for clinical intervention and further understanding of these processes.

No portion of this work has been published elsewhere or is under consideration by another journal. No financial or other interests exist which would create a conflict of interest for any of the author group, and all authors are in complete agreement with the manuscript contents. If accepted for publication, the authors agree to transfer all copyright ownership to the publisher.

Thank you very much for your ongoing consideration of our manuscript, which we hope is found suitable for publication with these additions. Please do not hesitate to contact me if further information is required.

Sincerely,

Michael D. Hogarty, MD
Professor of Pediatrics
Perelman School of Medicine at the University of Pennsylvania

Comments to the authors (excerpted to focus on concerns and questions): Note, we use the term MAM to represent both ERMCs (intra-organelle contacts) and MAMs (ERMCs isolated by fractionation) in our manuscript. Below we use whichever term was used in the query for clarity. We also refer to figures according to the EMBO J request, including Expanded View Figures (Fig.EVs).

Referee #1

Coku et al examine the role of ER-mitochondria contacts (ERMCs) in mediating cancer therapy resistance. They isolated mitochondria from neuroblastoma cell lines from the same patient at diagnosis (DX) and at relapse (REL). They observed that mitochondria from REL had markedly attenuated apoptotic responses and are resistant to diverse cancer therapeutics. Moreover, they attribute [this] to a reduction in ERMCs. The phenotype is not reverted by modulation of Ca^{2+} transfer between the two organelles but is (partially) reversed by KD of components of the ERMC: MFN2 and PACS2. They conclude that ER-mitochondria contact remodeling is required to acquire multidrug resistance but in a Ca^{2+} independent fashion.

While the study is of potential interest, I have a number of...methodological concerns, [and there is] a lack of a convincing mechanism for how alteration in ERMCs could contribute to therapy resistance. I will list all the major concerns, hoping to stimulate the authors to improve their investigations:

[1] The starting experiments measure mitochondrial sensitivity [for cyto c release] when stimulated with tBid or BimBH3 and reported that most REL lines developed resistance to cyto c release. They use heavy membrane fractions which contain mitochondria, MAMs, and other contaminants. This referee expects that ERMCs are not influential for MOMP execution [as] tBid and BimBH3 should be able to engage BAK regardless of the presence of ER. In this perspective, investigating ERMCs is not the most logical to explain the phenotype of Fig.1, and the lack of a role for Ca^{2+} transfer could be a confirmation of a lack of role for ERMCs. If authors want to focus on a role for tBid and BimBH3 in the induction of cyt C release in isolated mitos, then a robust molecular background should be offered.

Thank you for raising this important perspective, one that was initially shared by our authorship team as well. We probed heavy-membrane fractions with tBid or BimBH3 with the intent to directly measure their ability to oligomerize Bak or Bax and induce MOMP in mitochondria. We confirmed markedly reduced MOMP in REL tumor cells compared with patient-matched DX cells but did not find changes in Bak or Bax abundance, either in heavy-membrane fractions or whole cell lysates [(1); **new Fig.EV2** and **new text 232-234**]. Moreover, even saturating concentrations of tBid or Bim did not induce REL mitochondria to release cyto c to the extent released from DX mitochondria. To provide more mechanistic detail, we now show REL mitochondria have attenuated tBid induced oligomerization of Bak and Bax as compared with DX cells [more notable with Bak, the preferential tBid target (2)], providing mechanistic evidence the MOMP barrier occurs at the level of Bak/Bax oligomerization (**new Fig.1D and EV2**; **new text 172-177**). This was seen only in the DX/REL pairs that manifest multidrug resistance and not in the CHLA122/CHLA136 pair that does not, so we sought the reasons for this.

Oligomerization of Bak and Bax, and subsequent MOMP, is attenuated in REL tumor cell mitochondria in response to death stimuli yet we found no consistent changes in mitochondrial biomass, size, shape, or mtDNA abundance. However, we did identify reductions in ERMCs (or MAMs) in REL cells, including their frequency, gap-widths and lengths. Again, these ERMC differences were absent from our outlier DX/REL pair that did not manifest multidrug resistance. ERMCs have been postulated to play a role in supporting apoptotic signaling through the delivery of both calcium and bioactive lipids to mitochondria. We initially assessed calcium transfer and showed this was altered in ways predicted by the ERMC changes we identified (*further addressed in response 4, below*), but this did not account for changes in MOMP sensitization across our resistant DX/REL pairs.

To further explore how reduced ERMCs might attenuate Bak and Bax sensitivity to tBid we have now performed lipidomics on our DX/REL tumor pairs and implicate reduced ceramide synthesis and transfer as a principal mechanism. We find that REL cells with markedly depleted ERMCs (BE2C cells)

have increased sphingomyelins, reduced ceramides and elevated sphingomyelin:ceramide ratio (ERMCs are the site of sphingomyelinase-mediated hydrolysis of sphingomyelins to ceramides). REL cells with ERMC reductions limited to narrow gap-widths (termed lipid-ERMCs) like CHLA20 have markedly reduced ceramides also. The only REL cells absent such a reduction in ceramides is the CHLA136 cells that do not have a resistance phenotype. These data are detailed in **new Fig.6A-F** and **new Fig.EV5** and **new text 368-385**. We also now show that in therapy responsive DX cells, treatment of mitochondrial fractions with neutral sphingomyelinase inhibitors like GW-4896 disrupts this ceramide synthesis and transfer and phenocopies drug resistance and the attenuated MOMP responses seen in REL cells, respectively (**new Fig.6G-H** and **new Fig.EV2** and **new text 385-395**).

The lipid composition of the outer mitochondrial membrane (OMM) influences the recruitment of Bcl2 proteins as well as their conformational response to tBid and other Bcl2-family protein interactions [reviewed in (3, 4)]. Ceramide is synthesized by neutral sphingomyelinases at ERMCs and transferred to the OMM where it participates in the MOMP-driving pore formation governed by Bak and Bax. Ceramide itself can form pores in isolated mitochondria to induce cyto c release, though it synergizes with Bak/Bax to most effectively induce pore formation (5). Indeed, regulators of ceramide metabolism (including sphingomyelinase, the target of GW-4896) were identified as determinants of multidrug resistance using an unbiased siRNA screen across diverse carcinoma cell lines (6). That work was agnostic to any consideration of ERMC structure yet knock-down of 6 of 6 ceramide metabolizing enzymes led to drug sensitivity changes (increased or decreased) predicted by our ERMC-ceramide transfer model (6). Further, targeting neutral sphingomyelinases to various organelles has shown ceramide's effect on apoptosis requires its targeting to mitochondria (7) where it forms ceramide-rich macrodomains that enhance Bak or Bax oligomerization (8). Finally, mistargeting of the ceramide transport protein (CERT) to mitochondria enhances Bax-dependent MOMP and this requires a functional ceramide transfer domain (9). Here, we propose that ERMCs serve this "targeting" role to facilitate ceramide transfer to the OMM, and this is disrupted in REL tumor cells leading to resistance.

There is evidence that alterations in OMM ceramide content impacts cell survival in other pathophysiological states like cardiac ischemia-reperfusion injury (10). That injury is exacerbated by ceramide transfer at ERMCs and blocking neutral sphingomyelinase pharmacologically preserves cardiac function under this stress (10). Our findings are also consistent with work from Chipuk et al, who showed that MOMP induction requires the sphingolipid synthetic pathway, specifically the activity of neutral sphingomyelinases at ERMCs, and ceramide in the OMM (11). They also identified pivotal ceramide-derived lipids that enhance Bak and Bax activation and Walensky's group identified a binding site on Bax responsible for covalent lipid-mediated modulation of oligomerization potential (12). More recently, crystal structures have revealed Bak sites preferentially bound by lipids that are necessary to support oligomerization (13). This information has been added to the Discussion, **new text 484-517**. Collectively, these additions provide more detailed mechanisms between disrupted ERMCs and altered stress response in multidrug resistant cancer cells.

However, we recognize there are many additional questions that we have not yet answered. We have found the restoration of MOMP signaling in REL cell mitochondria to be challenging, despite efforts to provide ceramides and other bioactive lipids to the OMM, with or without the presence of relevant recombinant or purified lipid enzymes. The details of transfer between the ER and mitochondria are still largely unknown for sphingolipids. They are poorly soluble in aqueous compartments and much data supports a requirement for lipid transfer proteins in these processes, and these may not be present in our REL mitochondria with disrupted ERMCs. In support of this, much of the work demonstrating the impact of OMM ceramides in supporting Bak/Bax oligomerization and pore formation utilized the ectopic expression of ceramide transport proteins (9) or neutral sphingomyelinases (7) to the OMM.

[2] The choice of MFN2 and PACS2 does not guarantee the manipulation of ERMCs only. MFN2 KD is reported to modify mitochondrial morphology, which could impact apoptosis sensitivity, and PACS2 is reported to relocate to mitochondria during stress conditions to favor MOMP.

We agree with this limitation and yet most proteins implicated in the regulation of ERMCs serve additional roles in mitochondrial motility, fusion/fission dynamics, calcium metabolism, mitophagy, ER stress or related processes. To account for this, we sought independent proteins that are recurrently credentialed as regulators of ERMC initiation and/or maintenance, including a verified loss-of-ERMC phenotype in mammalian cells when knocked-down, and that had non-overlapping additional activities. *MFN2* and *PACS2* were selected according to these criteria, and we applied careful EM morphometry to characterize their impact on mitochondria when knocked-down. Mfn2 knock-down altered mitochondrial roundness and circularity but not size. Pacs2 knock-down increased mitochondrial size but not roundness or circularity (**Fig.4B**). With respect to functions that could confound our MOMP studies, Mfn2 is a fusion protein and yet inhibiting fusion (14) or inhibiting fission (15) in mitochondria have both been correlated with reduced tBid-mediated MOMP sensitivity. It is possible this discordance reflects changes in ERMCs under those perturbations (Mfn knock-out and a Drp-1 fission inhibitor, respectively) that were not assessed. As stated, in response to apoptosis inducers, Pacs2 helps translocate Bid to mitochondria and sensitizes toward MOMP. However, our cyto c release assays are done on isolated mitochondria without a preceding stressor and with our direct control over the delivery of exogenous tBid, so the reduction in Pacs2 should not interfere with our results. Importantly, in both of our knock-down models we show that not only is therapy resistance phenocopied, but the ERMC phenotype found in REL cells is recapitulated both by ERMC numbers (compare **Fig.3E** and **Fig.4F**) and gap-widths (compare **Fig.3F** and **Fig.4G**).

[3] A second critical point is that the study is merely descriptive and lacks a detailed molecular mechanism. It is obscure how therapy-resistant cancer cells could display lower ER-mitochondria contacts. Not even just expression levels of the ERMCs components are offered.

We agree that having a molecular mechanism is important but felt that the phenotype we identified, along with functional validation, was of sufficient novelty and impact to warrant submission for publication while we pursued more detailed mechanisms separately. However, we had the opportunity in this revision to address mechanisms more substantially, both by demonstrating the role of Bak and Bax oligomerization as an intermediate for MOMP downstream of death-stressors in our assays, and the role of disrupted ceramide transfer in attenuating Bak/Bax responsiveness (detailed above). We also assessed the expression of multiple putative ERMC proteins in our heavy membrane fractions yet were unable to demonstrate a reduction in ER or ERMC proteins in the REL cell fractions (**Fig.EV4F**). This likely represents the ubiquity of ER membranes (and ERMCs) that persist in our standard heavy membrane preparations despite their anatomic disruption and loss of functional integrity. While some have shown such protein levels can be surrogates for ERMC content, many in the organellar contacts field have failed to find this useful (16) and endorse EM morphometry as a more definitive measure. Only when we use more stringent mitochondria purification techniques such as antibody-based magnetic separation with column purification could we reliably show a reduction in ERMC proteins (e.g., Facl4 immunoblot in **new Fig.EV2E**).

[4] The authors stated that "reduced transfer of Ca^{2+} from ER to mitochondria is not required for mitochondrial desensitization towards apoptotic MOMP". They perform mitochondrial calcium measurement in two different DX/REL pairs obtaining opposite results; it is not clear how they explain these data. In front of the strong reduction in ERMCs described in Figure 3, a strong alteration in Ca^{2+} transfer is just expected. How do authors explain this observation? Coupling time is not the most used readout to verify this phenotype, while the elevation of $[Ca^{2+}]m$ is the most expected but not observed. Again, how could authors explain this phenotype? Also, augmentation of ERMCs should favor cyto c release regardless of Ca^{2+}, but this measurement was not reported.

Indeed, we also expected that all our REL drug resistant cells with reduced ERMCs would manifest reduced Ca^{2+} transfer. However, we confirmed this only in BE2C (REL) but not in CHLA20 (REL) cells when compared with patient-matched DX cells. EM morphometry showed their ERMC

phenotypes differed, with the latter having lost ERMCs at close gap-widths (<10 nm) with preservation of larger gap-width ERMCs (**Table 1**). Ca^{2+} release at ERMCs create high concentration microdomains that promote uptake via the low-affinity mitochondrial Ca^{2+} uniporter, with most efficient transfer occurring across a gap-width of 10-25 nm (17). BE2C cells are markedly deficient in ERMCs within 25 nm, whereas CHLA20 cells maintain ERMCs at 10-25 nm but are markedly reduced at <10 nm. We propose this explains why Ca^{2+} transfer is maintained in CHLA20 but not BE2C, while lipid transfer is disrupted in both as the latter requires closer contacts (18). This has been clarified in the revised manuscript (**new text 470-489**) and in the revised schematic in **Fig.7**. We also show that disrupting ceramide but not Ca^{2+} at ERMCs attenuates MOMP.

We and others have investigated a variety of parameters to assess the local Ca^{2+} transfer from ER to mitochondria. Based on our experience, the coupling time is the best parameter for this when both cytosolic and mitochondrial matrix [Ca^{2+}] are recorded simultaneously. The elevation in mitochondrial [Ca^{2+}] sometimes involves a slow component that is not necessarily supported by ERMC transfer so we prefer to continue to use the coupling time for this, as presented in the manuscript.

[5] The authors stated that immunomagnetic-bead separation induced less membrane disruption, and MOMP sensitivity in response to BimBH3 was greater for CHLA15 heavy membrane fractions (mitochondria with intact MAMs) compared with purified mitochondria (reduced MAMs; Figure 5A). They should demonstrate the different MAMs amount after this separation.

We now confirm that both limited proteolysis and MACS-based mitochondrial purification techniques have minimal impact on mitochondrial Bak and Bax content or integrity by immunoblot (new **Fig.EV2D-E**). We also assessed the abundance of the ERMC marker, fatty-acid Co-A ligase 4 (Facl4). MACS-based purification significantly reduced the abundance of Facl4, yet this remained detectable supporting that purification remained incomplete, as has been reported by others. Still, this partial reduction was sufficient to largely phenocopy the attenuated cyto c release seen in REL mitochondria (**Fig.4A**). We did not perform EM analyses since these are all performed on heavy membrane fractions rather than intact cells where ERMC morphometry is most informative.

Referee #2

Coku and colleagues investigate the basis for multidrug resistance in neuroblastoma. Using matched [cell] lines pre/post treatment (sensitive and resistant) they use BH3-profiling to demonstrate a defect in mitochondrial apoptosis...they propose defective mito-ER contacts (MAMs [or ERMCs]) as the basis for defective apoptosis induction and drug-resistance. Various earlier studies have made links between MAMs and apoptotic sensitivity, prominently by Ca transfer (tested here) or sphingolipid metabolism. The main novelty in this study, in my opinion, centers on the proposal that MAMs are important for drug-sensitivity in neuroblastoma and that loss of MAMs can be selected for as a means of drug resistance. Nevertheless, I consider a definitive causative role for loss MAMs in drug resistance needs further validation as does why loss may contribute to drug resistance - mechanistically this remains unclear.

[1] Further demonstration of the importance of loss of MAMs in the effects described is required (Figure 5) - the limited proteolysis expt. (disrupting MAMs) is very difficult to control due to possible cleavage of proteins involved in MOMP (e.g BAK) leading indirectly to inhibitory effects on MOMP; PACS2 and MFN2 knockdown can of course have a lot of indirect effects, since both proteins have various purported functions. One prediction of the model would be that direct tethering of the ER to mitochondria would bypass apoptotic resistance in the relapse cells - this can be directly addressed using the excellent tools co-author Hajnockzy has previously made (PMID 20603080).

These are valid concerns that we have now directly address. We also should clarify a minor error in our manuscript: to deplete MAMs from mitochondria we tested both limited tryptic digestion and MACS-based separation and the latter was more successful for our downstream cyto c release assay.

However, our prior **Fig.5** legend stated “CHLA15 were rendered MAM-depleted by partial proteolysis *and* immune-magnetic cell sorting…”. This is inaccurate, as we used mitochondria purified only with immune-magnetic cell sorting (and absent proteolysis) for all experimental data shown. The text and M&M sections were correct, and this legend has now been corrected (**now as Fig.4**).

Limited proteolysis is more likely to alter OMM-embedded proteins. Though we explored both methods, the data in the manuscript (and this revision) showing that MAM depletion attenuates MOMP used MACS separation (immuno-magnetically purified using TOM22 antibodies) that is less likely to disrupt Bak or Bax. We assessed the impact of both limited proteolysis and MACS and neither technique reduced Bak or Bax or created cleavage isoforms (**new Fig.S2D-E**). Please also see Response 2 and 4 to Referee 1 (above) for responses related to *MFN2* and *PACS2* knock-down.

As also mentioned, synthetic linkers can be used to re-approximate ER with the OMM. This allows linker enhanced MAMs to be directly identified via imaging, and their impact on calcium transfer measured, as we show in **Fig.5**. However, the relatively low transfection efficiency and the resultant hypersensitivity to stress such MAM tightening induces makes it extremely difficult to stably select and study such cells for their response to exogenous stressors like chemotherapy. The other theoretical problem with this approach is that synthetically approximating ER with the OMM does not necessarily restore the lipid synthesis and/or transfer functionality of a native cellular ERMC.

[2] In addition to these experiments, the effects of modulating MAM numbers on therapy induced death (agents used in Fig 2) should also be addressed, to determine the relevance of MAM in clinically relevant neuroblastoma therapies.

We have done this with a drug that is a better assessment for this phenotype as it directly engages Bak/Bax-mediated MOMP, the Bcl2 inhibitor, ABT737. Neuroblastoma cells are dependent on the basal repression of endogenously activated Bim by *either Bcl2 or Mcl1*. In Bcl2-dependent cells like CHLA15 we show Bim is sequestered by Bcl2 in viable proliferating tumor cells, while ABT737 displaces Bim from Bcl2 leading to Bim-mediated Bak/Bax oligomerization, MOMP, and cell death at nanomolar sensitivity (1). This drug is therefore operating directly upon the relevant mechanism impacted by reduced MAMs without any confounding mechanisms operative upstream. It is also a relevant therapeutic as a Phase 1/2 trial of the Bcl2 inhibitor venetoclax is enrolling an expansion cohort of children with neuroblastoma on the basis of data from our lab and others (NCT03236857).

[3] How MAMs contribute to apoptotic sensitivity here is not clear, the authors extensively investigated a role for Ca transfer (finding none), one other possibly based on the work by Chipuk and colleagues (discussed at various points) is through effects on sphingolipid generation/mitochondrial transfer supporting pro-apoptotic BAX activation, this should be investigated.

We agree. While we maintain that the MAM-reduced phenotype we identified as a driver of multidrug resistance is novel and impactful in its own right, providing a mechanistic underpinning markedly enhances its importance. We hypothesized that disruptions in the lipid signals mitochondria derive from MAMs leads to an altered lipid milieu in the OMM, which in turn attenuates Bak/Bax responsiveness. Elegant work done by Chipuk and Green and colleagues, among others, support this (4, 5, 7-9, 11) though it has never been demonstrated as a mechanism of acquired therapy resistance in cancer. We now provide evidence for this in our revised manuscript. *Please see Responses 1 to Referee 1 above for further details*. Note, while we have obtained clear evidence for the contributions of disrupted ceramide generation from ERMCs, and the impact of inhibiting ceramide synthesis on therapy resistance, it is also fair to say that many questions remain. Inter-organelle contactology is a nascent field and the relationships among ERMC morphologies, dynamics and functions are still being elucidated. We propose that this work is an important contribution that will enable further studies.

[4] An obvious point of apoptotic deregulation is through upregulation of anti-apoptotic BCL-2 family members, the authors have previously discounted this in another study, this should also be highlighted where they state "loss of BAX or BAK was unlikely...."

Yes, we agree that stating this will enhance the clarity of the manuscript and have added this information as recommended (**new text 232-234**). In agreement with this is our data that saturating concentrations of tBid or BimBH3 fail to induce the same proportion of cyto c release from REL cells compared with DX cells. Such a profound release barrier argues for a principal defect in Bak/Bax signaling as we show, since such high concentrations of death-effectors would saturate upregulated anti-apoptotic protein binding sites and still engage Bak or Bax. In addition, we have also reconfirmed stable Bak and Bax in both whole cell lysates and in heavy membrane fractions from the DX and REL tumor pairs used in our work (**new Fig.EV2**, and Response 1 to Reviewer 1).

Referee #3

Interesting paper linking therapy resistance in relapsed patients to reduced endoplasmic reticulum-mitochondrial contacts. Quality of science is good, but all studies [are] done in vitro and sample sizes are quite small.

We appreciate the complimentary statement and acknowledge the limitations. The field will certainly benefit from improved and standardized methods to quantify the geometry and extent of MAMs in cells and tissues. We utilized an EM morphometric approach as it provides high-nanoscale resolution images. While low throughput, we used it to characterize the architecture of >2,400 discrete MAMs in patient-matched cancer cells at the time of diagnosis and after relapse to identify this novel resistance phenotype. A more extensive understanding of the mechanisms leading to multidrug resistance will enable in vivo studies that seek to leverage these findings to enhance therapy responsiveness, and efforts to restore ceramide delivery to mitochondria, as identified herein, provides one such opportunity.

[1] The authors did show that exposure of cells in vitro to ABT737 led to acquired resistance. Do these cells acquire reduced MAMs?

In our work looking at acquired resistance to Bcl2 inhibitors like ABT737 we find that in all instances, neuroblastoma cells that were sensitive (i.e., were utilizing Bcl2 to sequester activated Bim) became resistance by switching to Mcl1 to sequester Bim (1). They did not acquire a multidrug resistant phenotype nor altered MOMP sensitivity when using cyto c release assays. Drugs applying a very selective or targeted stress may provide more immediate escape or resistance opportunities, as in this instance, than do modern era multimodality therapies that induce broad genotoxic and metabolic insults.

[2] Can any conclusions about reduced ERMCs in response to therapy be associated with genetic aberrations in the neuroblastoma?

The acquisition of multidrug resistance in neuroblastoma is not associated with acquired changes in credentialed drivers such as *MYCN*, *ALK*, *MAPK* genes or *TP53* either in our tumor samples or more broadly. We have also performed a standard NGS-based cancer panel on these tumors and did not identify any consistent genetic alteration that correlated with this phenotype. Our bias is that this phenotype reflects a non-genetic resistance mechanism and may be correlated with cell lineage plasticity, though our ongoing exploration of this is beyond the scope of the current manuscript. A deeper understanding of what regulates how MAMs are formed, reshaped, maintained and/or removed within cells may provide additional insight into mechanisms whereby MAMs may be selectively depleted in a cancer cell, and what the fitness cost of such depletion would be. This should also provide a better foundation for more directly interrogating these pathways for genetic mutation in the future.

Citations:

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Dear Dr Hogarty,

Thank you for submitting your revised manuscript (EMBOJ-2021-108272R) to The EMBO Journal. Your amended study was sent back to the three referees for re-evaluation, and we have received comments from two of them, which I enclose below. Please note that while referee #3 was at this time not able to provide us with a re-report, we have editorially assessed your response to his-her critique, and found it to be considered in a satisfactory manner. As you will see, the other referees stated that the issues raised have been adequately addressed and they are broadly now in favour of publication, pending a minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining points of referee #1 carefully, and address these by either adding complementary data or introducing caveats where appropriate.

In addition, we need you to take care of a number of issues related to formatting and data representation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please add the accession number for the lipidomics data set to the data accessibility section, and make sure the privacy is released.

>> Amend the author checklist with the accession number for the lipidomics data set.

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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 recommend a revision within 3 months (18th Mar 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

The authors have significantly improved the manuscript and successfully addressed the most critical points that I originally raised. The manuscript now does sufficiently supports the proposed model and I support its positive evaluation by the Editor.

A couple of minor points should still be addressed:
- Figure 1D and EV2. The loading controls appear significantly uneven between samples. The author should include a relative quantitation of the protein of interest on the loading control (e.g. for F1D the integrated density of BAK oligomers on the integrated density of VDAC).
- Figure 5A. The authors did not explain why the coupling time should be a preferable readout of impaired Ca2+ transfer. As in my opinion, this readout is hard to understand, I strongly recommend supporting it with an index of the amount of Ca2+ mobilized by the agonist in the mitochondrial matrix and cytoplasm (ideally the maximal amplitude of agonist-induced Ca2+ peak).

Referee #2:

The authors have comprehensively addressed all comments I raised - I appreciate their efforts.

Dear Dr Hogarty,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

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Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We would appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update

of the entry status. Thank you in advance!

If you have any questions, please do not hesitate to call or email the Editorial Office.

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

Kind regards,

Daniel Klimmeck

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Corresponding Author Name:	Michael D. Hogarty
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

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The data shown in figures should satisfy the following conditions:

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

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Our work involved at least 3 biological replicates in which 2-3 technical replicates each were performed. The assays are low throughput and not done with prior power calculations or effect size so descriptions of all data are showed in figures and statistically analyzed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For cytochrome c release assays, a QC step was to ensure non-specific release of cytochrome c was <30% of available cytochrome c, as mechanical dysruption of the OMM could cause non-specific release. This was defined a priori and has been used as a QC step in all such assays.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For mito characteristics (size, circularity, etc) and DNA damage assessment we used a non-parametric statistical test rather than transform the data that is non-normally distributed (Mann Whitney U test). We used the Kolmogorov-Smirnov test to assess for normality. However, some of our data is low throughput and with small numbers of replicates, these were analyzed by the student t-test since there was no clear violation of the normal distribution requirement.
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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Lipidomics data is publicly available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website: Study ID ST002054 (Project DOI: http://dx.doi.org/10.21228/M8T13T)
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