EMBO Molecular Medicine

Dantrolene corrects cellular disease features of Darier disease and may be a novel treatment

Matthew Hunt, Nuoqi Wang, Naricha Pupinyo, Philip Curman, Monica Torres, William Jebril, Maria Chatzinikolaou, Julie Lorent, Gilad Silberberg, Ritu Bansal, Teresa Burner, Jing Zhou, Susanne Kimeswenger, Wolfram Hötzenecker, Keith Choate, Etty Bachar-Wikstrom, and Jakob Wikstrom

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Review Timeline:

Submission Date:	5th Jun 23
Editorial Decision:	4th Jul 23
Revision Received:	30th Jan 24
Editorial Decision:	4th Mar 24
Revision Received:	5th Mar 24
Editorial Decision:	7th Mar 24
Revision Received:	26th Jun 24
Accepted:	4th Jul 24

Editor: Zeljko Durdevic

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.)

4th Jul 2023

Dear Dr. Wikstrom,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. All three referees recognize potential interest of the study but also raise important and partially overlapping criticism that should be addressed in a major revision. For further consideration of the manuscript, it is necessary to repeat essential experiments in patient keratinocytes or to at least show that the knockdown of ATP2A2 in primary keratinocytes resembles the cellular disease pathology. Experiments using animal model of Darier disease and addition of Hailey-Hailey disease patients as suggested by the referee #1 are not required and should be addressed by discussion in the manuscript. Further, I would like you to consider reformatting your manuscript to a scientific report type of article (3 figures, ~22000 characters), for more information please check our "Author Guidelines".
https://www.embopress.org/page/journal/17574684/authorguide#reportsarticleguide

If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

*****

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines

(https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

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In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- 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.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

See detailed instructions here:

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12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations,

relevant databases, OMIM/proteins/genes links, author's websites, etc...

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Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

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***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

In Darier disease the keratinocytes are skin cells that are mostly affected by the loss of function mutations of the SERCA pump. However, the authors selected HEK cells and for some of the experiments patient-derived fibroblasts as a working model. I do not fully understand why were keratinocytes not used at least for some of the experiments. Confirming the findings in an animal model of DD plus and minus Dantrelone would increase the clinical relevance of the study.
Since Danrelone is in clinical use, the authors might want to search patient databases for individuals treated with dantralone and evaluate how this might have affected gene expression or protein abundance in the skin or in outer organs. This might be helpful in enhancing the relevance of the mechanisms identified in HEK cells.

Referee #1 (Remarks for Author):

In this manuscript the authors propose Dantrolene sodium (D1) as potential drug in treating patients with Darier disease caused by mutations in the SERCA pump (ATP2A2).
Indeed, this is an interesting subject as novel cures for DD as well as Hailey-Hailey disease (HHD) are very much needed. However, the study needs significant improvement and additional experimental investigation in order to prove if Dantrolene can be used in treating DD and HHD.

I have several suggestions and comments:
1. Given that keratinocytes are most affected cells the authors should try to identify gene alterations only in these cells (experimentally or at least bioinformatically). Here, I refer to the experiments depicted in Fig 1 and S1. It is very likely that the gene expression in the biopsy samples are strongly affected by fibroblasts, melanocytes and even immune cells of the skin.
2. Referring to Fig. 2, additional calcium measurements are needed. Given the importance of ER Ca2+ signaling, it is very likely that the cytosolic as well as mitochondrial homeostasis will be affected in cells with non-functional SERCA. Also more detailed information about the ensor used to measure ER calcium is needed. For this study using a ratiometric one is essential. Normalizing the values to 1 leads to loss of a very important information i.e. the resting levels before stimulation, which are very likely also altered.
3. The study would profit if patients with HHD would be involved.
4. Regarding the study design and selection of experimental tools please see my comments in section 4.
5. In experiments in which Thapsigargin was used the authors should consider the fact that Tg induces ER stress.
6. Given that SERCA inactivity leads to ER calcium depletion, the STIM-ORAI calium entry machinery (already indicated as prognostic markers for DD and HHD) is most likely activated in these cells (keratinocytes). This would also mean that NFAT is

more active and that many of the genes altered in the DD patients are under control of NFAT. Is Dantrolene affecting NFAT activity?
7. The mitochondrial contribution requires further examination.

Minor.
HEK cell treated with siRNA or similar should not be referred to as disease model.
the figure call-outs should be checked. For example Fig. 2G is mentioned before Fig. 2A-E.
A full list of genes from the RNAseq with the corresponding fold change and P vallues should be provided.

Referee #2 (Comments on Novelty/Model System for Author):

This reports repurposes dantrolene, used to treat muscle spasticity or malignant hyperthermia, to treat the impaired endoplasmic reticulum calcium storage and defective cell-to-cell adhesion associated with Darier's skin disease. The experiments are carefully done. More experimental detail could be added, as sometimes it is difficult to understand the experimental approach. In addition, the conclusion that clinical trials are the next step appears to be overstated, as dantrolene only appears to be effective in reversing functional defects only in low doses of thapsigargin. However, these criticisms should be relatively easily addressed by the authors.

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Specific Criticisms:
Methods:
1) For genetic analysis, were whole skin samples or samples containing only epidermis analyzed. If whole skin samples, why was dermis included since the pathology appears to be in the epidermis only?
2) Cell culture: what was the calcium concentration in the cell culture medium?
3) Calcium measurements: please include what solutions were used for calcium measurements. If media was used as the solution, were the solutions gassed with appropriate CO2?
Fig 2. Its difficult to understand exactly what happened in this figure without going back to the methods. Please add this information in the figure legend.
Figure 5. There appear to be variable responses for everything except CHOP. Explanation?
Figure 6. Dantrolene appears to be effective only at TG 10 nM dose fir cell viability. Do the authors know what its effect is on ER calcium in micromolar concentrations? Figure 2 only shows a normalized fluorescence intensity.
Discussion: the authors state that there have been no clinical trials for Darier's disease. It's more accurate to say there have been no successful trials. Also its worth discussing whether the changes in adhesion and apoptosis are large enough in cell culture to warrant a clinical trial.

Referee #3 (Comments on Novelty/Model System for Author):

The authors chose to use siRNA knockdown of ATP2A2 in primary keratinocytes despite ready access to darier patients - knockdown is not the same as at the haploinsufficiency seen in DD patients. It's not clear that the model is true to the disease state. Furthermore, use of DD fibroblasts is odd given the absence of clear pathology in these cells in human disease.

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It is not surprising that a calcium channel inhibitor mitigates the effects of decreased ER calcium stores in DD..

The authors should cite manuscripts showing the IL17 inhibition has efficacy in DDD.

Figure 2: The authors appear to be seeing caclcium retention in excess of normal keratinocytes (siNEG/DL), nearly 2-fold greater. Normalization would be expected to be more relevant. Panels C and D are from non-relevant cells and have expected results. E appear to have a typo on right upper panel. While confocal is interesting - graphical presentation of analysis of multiple cells would be of greater benefit. F - it is not clear why fibroblast data is presented.

Given suggestion of therpeutic effect, have the authors considered RNA sequencing of normal keratinocytes, their atp2a2 knockdown cells, and the same cells treated with DL to show global normalization of gene function.

Overall, my greatest concern is the use of a non-physiologic model for DD. While the findings presented may be true, it is equally possible that they do not reproduce disease features of DD.

Comments from the editor:

“For further consideration of the manuscript, it is necessary to repeat essential experiments in patient keratinocytes or to at least show that the knockdown of ATP2A2 in primary keratinocytes resembles the cellular disease pathology. Experiments using animal model of Darier disease and addition of Hailey-Hailey disease patients as suggested by the referee #1 are not required and should be addressed by discussion in the manuscript.”

Answer: Firstly, we would like to thank you for considering our study for resubmission. We have, as suggested, repeated several experiments in patients’ keratinocytes (as well as performed other experiments as outlined below). Regarding Hailey-Hailey we address this disease in the Discussion.

“Further, I would like you to consider reformatting your manuscript to a scientific report type of article (3 figures, ~22000 characters), for more information please check our "Author Guidelines".”

Answer: The manuscript contains a considerable amount of data, especially after all the revision experiments requested, and it is not possible for us to cut it. We hope you have understanding for this.

Reviewer's comments

Referee #1 (Comments on Novelty/Model System for Author):

In Darier disease the keratinocytes are skin cells that are mostly affected by the loss of function mutations of the SERCA pump. However, the authors selected HEK cells and for some of the experiments patient-derived fibroblasts as a working model. I do not fully understand why were keratinocytes not used at least for some of the experiments. Confirming the findings in an animal model of DD plus and minus Dantrelone would increase the clinical relevance of the study. Since Danrelone is in clinical use, the authors might want to search patient databases for individuals treated with dantralone and evaluate how this might have affected gene expression or protein abundance in the skin or in outer organs. This might be helpful in enhancing the relevance of the mechanisms identified in HEK cells.

Answer: We would like to thank the reviewer for investing time in reviewing our manuscript. With regards to the first point made, we would like to clarify that we used HEKa cells, which are primary keratinocyte cells, either with pharmacological or genetic SERCA2 inhibition. With regards to the point about animal models of DD, we did not use one as there are no validated animal models currently established which recapitulate the pathophysiology of DD (the knockout mice have a completely different phenotype, they get skin cancer instead of the typical DD rash). Instead, we have performed new experiments in patient derived keratinocytes (**Figures 2-5**). And finally, with regards to the point about patient databases, we have been unable to find any case reports about the use of Dl in DD patients, and so believe there not to be any.

Referee #1 (Remarks for Author):

In this manuscript the authors propose Dantrolene sodium (D1) as potential drug in treating patients with Darier disease caused by mutations in the SERCA pump (ATP2A2).
Indeed, this is an interesting subject as novel cures for DD as well as Hailey-Hailey disease (HHD) are very much needed.
However, the study needs significant improvement and additional experimental investigation in order to prove if Dantrolene can be used in treating DD and HHD.

I have several suggestions and comments:
1. Given that keratinocytes are most affected cells the authors should try to identify gene alterations only in these cells (experimentally or at least bioinformatically). Here, I refer to the experiments depicted in Fig 1 and S1. It is very likely that the gene expression in the biopsy samples are strongly affected by fibroblasts, melanocytes and even immune cells of the skin.

Answer: In the original experiment, we tested the extraction of RNA from epidermis only, dermis only, and epidermis + dermis and found that the RIN values from dermis samples were negligible (thus indicating that little dermis was included due to careful dissection). As such, in order to avoid altering the samples by lengthy enzymatic removal of the dermis from the epidermis, we reasoned that using epidermis + dermis samples was most appropriate. With regard to the reviewer's comments, we performed deconvolution analysis of the bulk RNAseq data through the xCell tool and found that there was negligible amounts of fibroblasts in the samples used, and that the main population of cells was keratinocytes (**Figure EV 1C**). In addition, through this analysis we were also able to determine that there were very few significant differences in cell type composition between lesional and non-lesional biopsies. Regarding Hailey-Hailey disease, please see our answer to point 3 below.

2. Referring to Fig. 2, additional calcium measurements are needed. Given the importance of ER Ca2+ signaling, it is very likely that the cytosolic as well as mitochondrial homeostasis will be affected in cells with non-functional SERCA. Also more detailed information about the ensor used to measure ER calcium is needed. For this study using a ratiometric one is essential. Normalizing the values to 1 leads to loss of a very important information i.e. the resting levels before stimulation, which are very likely also altered.

Answer: In response to the reviewer's helpful comments, we have added a graphical representation to summarise the experimental setup (**Figure 2A**), as well as adding more detailed text about the experimental methods in a new 'Ca^{2+} imaging' section in the methods. In addition, as well as the ER-Ca^{2+} analysis, we have additionally performed experiments to analyse cytosolic calcium dynamics using the ratiometric dye Fura Red AM, as well as mitochondrial calcium through live cell imaging of Rhod-2 AM.

3. The study would profit if patients with HHD would be involved.

Answer: Whilst we agree with and appreciate the comment about the benefit of including HHD patients in the study due to the similarities between HHD and DD, we feel that studying the effect of Dl or another novel treatment in the context of HHD should be performed separately, as in this study we aimed to specifically investigate the effect on SERCA2 and therefore ER, not Golgi. We plan to study HHD in the near future, but as the pathophysiology is less understood than DD at presence, we felt that this was not in the scope of this manuscript.

4. Regarding the study design and selection of experimental tools please see my comments in section 4.

Answer: We hope that we have addressed this comment in the reply to point 2.

5. In experiments in which Thapsigargin was used the authors should consider the fact that Tg induces ER stress.

Answer: We appreciate the comment. Tg is a well-known inhibitor of SERCA2 and so is widely used in studies investigating DD, in which ER stress is also an acknowledged feature. As such, ER stress was an accepted consequence of using Tg in our study, and ER stress was measured in Tg-treated HEKa cells (**Figure 4B**). In addition, we note that incubation may be an issue in experiments with longer incubation times, but in our experiments we used a low concentration of Tg and only for 24 hours. Finally, as patient keratinocytes has been included the manuscript relies less on the Tg model.

6. Given that SERCA inactivity leads to ER calcium depletion, the STIM-ORAI calium entry machinery (already indicated as prognostic markers for DD and HHD) is most likely activated in these cells (keratinocytes). This would also mean that NFAT is more active and that many of the genes altered in the DD patients are under control of NFAT. Is Dantrolene affecting NFAT activity?

Answer: We thank the reviewer for this helpful comment. In response, we assessed the gene expression of various NFAT markers as well as downstream targets of significance through RT-qPCR and found that Dl reduced the expression of *NFAT1* and *NFAT4* in HEKa cells. In addition, we found that NFAT expression was significantly higher in mutant keratinocytes compared to control, and that there was a trend towards Dl decreasing expression of various NFAT-related genes in the mutant DDK patient cells (**Figure EV 3**). These findings are mentioned in the Results as well as Discussion.

7. The mitochondrial contribution requires further examination.

Answer: We agree that the contribution of mitochondria in DD is interesting and requires further examination. However, we feel that investigation of this requires significant work and so is not in the scope of this paper. Indeed, we plan to investigate this thoroughly in a separate study which is part of PhD student project in our lab.

Minor.

HEK cell treated with siRNA or similar should not be referred to as disease model.

Answer: We agree with this comment and as such have changed all references to Tg-treated HEKa or si*ATP2A2* HEKa as models of SERCA2 inhibition, with primary DD keratinocytes now only being referred to as disease models.

The figure call-outs should be checked. For example Fig. 2G is mentioned before Fig. 2A-E.

Answer: We appreciate the raising of this comment and have address it throughout the manuscript.

A full list of genes from the RNAseq with the corresponding fold change and P vallues should be provided.

Answer: We agree with this comment and as such have included the full list of RNAseq genes and their differential expression analysis to the manuscript (**Appendix Figure S1**).

Referee #2 (Comments on Novelty/Model System for Author):

This reports repurposes dantrolene, used to treat muscle spasticity or malignant hyperthermia, to treat the impaired endoplasmic reticulum calcium storage and defective cell-to-cell adhesion associated with Darier's skin disease. The experiments are carefully done. More experimental detail could be added, as sometimes it is difficult to understand the experimental approach. In addition, the conclusion that clinical trials are the next step appears to be overstated, as dantrolene only appears to be effective in reversing functional defects only in low doses of thapsigargin. However, these criticisms should be relatively easily addressed by the authors.

Answer: We thank the reviewer for the possible outlook on the manuscript and for taking the time to review it. In response to the reviewer's helpful comments, we have added a graphical representation to summarise the experimental setup (**Figure 2A**), as well as adding more detailed text about the experimental methods in a new 'Ca^{2+} imaging' section in the methods. In addition, as well as the ER-Ca^{2+} analysis, we have additionally performed experiments to analyse intracellular calcium dynamics using the ratiometric dye Fura Red AM, as well as mitochondrial calcium through live cell imaging of Rhod-2 AM (**Figure 2B-G**). With regards to their comment regarding the statement about clinical trials, we hope that including additional experimental data using primary DD keratinocytes will support our observation that clinical trials of Dl on DD patients are appropriate.

Referee #2 (Remarks for Author):

General remarks: This reports repurposes dantrolene, used to treat muscle spasticity or malignant hyperthermia, to treat the impaired endoplasmic reticulum calcium storage and defective cell-to-cell adhesion associated with Darier's skin

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Answer: Please see the previous answer (the comment is duplicated).

Specific Criticisms:
Methods:
1) For genetic analysis, were whole skin samples or samples containing only epidermis analyzed. If whole skin samples, why was dermis included since the pathology appears to be in the epidermis only?

Answer: We appreciate this point being raised by the reviewer. In the original experiment, we tested the extraction of RNA from epidermis only, dermis only, and epidermis + dermis and found that the RIN values from dermis samples were negligible (thus indicating that little dermis was included due to careful dissection). As such, in order to avoid altering the samples by lengthy enzymatic removal of the dermis from the epidermis, we reasoned that using epidermis + dermis samples was most appropriate. With regard to the reviewer's comments, we performed deconvolution analysis of the bulk RNAseq data through the xCell tool and found that there was negligible amounts of fibroblasts in the samples used, and that the main population of cells was keratinocytes (**Figure EV 1C**). In addition, through this analysis we were also able to determine that there were very few significant differences in cell type composition between lesional and non-lesional biopsies. Regarding Hailey-Hailey disease, please see our answer to point 3 below.

2) Cell culture: what was the calcium concentration in the cell culture medium?

Answer: We have answered this in the Methods section 'cell culture and treatments'.

3) Calcium measurements: please include what solutions were used for calcium measurements. If media was used as the solution, were the solutions gassed with appropriate CO2?

Answer: Culture media was used and yes, it was gassed with appropriate CO2. As mentioned in response to point 2 from reviewer 1, we have included more extensive details about the experimental setup and conditions used in the calcium measurement experiments in the 'Ca^{2+} imaging' section in the methods section, including details about the gas conditions during imaging.

Fig 2. Its difficult to understand exactly what happened in this figure without going back to the methods. Please add this information in the figure legend.

Answer: We agree with the point raised, and as such, have included a graphical cartoon to the figure to summarise the experimental details (Figure 2A). In addition, we have included more detail in the results section.

Figure 5. There appear to be variable responses for everything except CHOP. Explanation?

Answer: It appears that the rescue effect of Dantrolene on ER stress is mostly through CHOP in the keratinocytes, which might explain the reduction in apoptosis with dantrolene. The rest of the UPR is largely unaffected in keratinocytes however in patient fibroblasts overall UPR is reduced with dantrolene treatment. We elaborate on this in the Discussion section on UPR. Furthermore, please note that the ER stress figure now is Figure 4 and that we have added data on patient derived keratinocytes.

Figure 6. Dantrolene appears to be effective only at TG 10 nM dose fir cell viability. Do the authors know what its effect is on ER calcium in micromolar concentrations? Figure 2 only shows a normalized fluorescence intensity.

Answer: No, measured calcium in relative units and therefore we do not know the concentrations in micromolar, but we substantially expanded our calcium measurements by analysis of cytosolic calcium dynamics using the ratiometric dye Fura Red AM, as well as mitochondrial calcium through live cell imaging of Rhod-2 AM (**Figure 2**). These experiments were also expanded to patient derived keratinocytes. Furthermore, regarding cell viability and thapsigargin concentrations, cell viability is a less sensitive assay than apoptosis which did show dantrolene rescue at high thapsigargin concentrations (**Figure 5F**).

Discussion: the authors state that there have been no clinical trials for Darier's disease. It's more accurate to say there have been no successful trials. Also its worth discussing whether the changes in adhesion and apoptosis are large enough in cell culture to warrant a clinical trial.

Answer: We acknowledge the reviewers first comment and have addressed this to say that there have been no successful clinical trials (Discussion). With regards to the second point, as there are no validated animal models for DD, we feel that our statement about the need for clinical trials with DI is warranted in the face of our *in vitro* data. Moreover, such a trial would be based on drug repurposing of a safe drug that has been in use since the 1970's.

Referee #3 (Comments on Novelty/Model System for Author):

The authors chose to use siRNA knockdown of ATP2A2 in primary keratinocytes despite ready access to darier patients - knockdown is not the same as at the haploinsufficiency seen in DD patients. It's not clear that the model is true to the disease state. Furthermore, use of DD fibroblasts is odd given the absence of clear pathology in these cells in human disease.

Answer: First, thanks for your thorough review. We have addressed this by performing experiments on patient derived primary keratinocytes (**Figure 2-5**).

Referee #3 (Remarks for Author):

The authors chose to use siRNA knockdown of ATP2A2 in primary keratinocytes despite ready access to darier patients - knockdown is not the same as at the haploinsufficiency seen in DD patients. It's not clear that the model is true to the disease state. Furthermore, use of DD fibroblasts is odd given the absence of clear pathology in these cells in human disease. This data is not a surrogate for studying patient keratinocytes.
It is not surprising that a calcium channel inhibitor mitigates the effects of decreased ER calcium stores in DD..
The authors should cite manuscripts showing the IL17 inhibition has efficacy in DDD.

Answer: We thank the reviewer for their time reviewing our manuscript. With regards to their first point, we acknowledge that KD of *ATP2A2* is not the same as DD, and so have corrected this throughout the paper to state that the si*ATP2A2* HEKa cells are models of genetic SERCA2 inhibition. For the second point, whilst we agree with the point about DD fibroblasts, we feel that their inclusion is of some interest, due to the fact that DD pathogenesis affects several cell types and organs, although clearly not the same extent as keratinocytes. With regard to this, we have repeated essential experiments in primary DD keratinocytes (**Figure 2-5**) and kept the DD fibroblast data, although placed less importance to it. Regarding the effect of a calcium channel inhibitor, we agree that this is logical, and this is exactly why we chose to perform this study. It is a bit surprising that this has not been tested before, but bear in mind that Darier disease is quite rare and only studied by a few researchers. Finally, we agree with the suggestion about IL17 inhibition and have included text discussing this in the Discussion.

Figure 2: The authors appear to be seeing caclcium retention in excess of normal keratinocytes (siNEG/DL), nearly 2-fold greater. Normalization would be expected to be more relevant. Panels C and D are from non-relevant cells and have expected results. E appear to have a typo on right upper panel. While confocal is interesting - graphical presentation of analysis of multiple cells would be of greater benefit. F - it is not clear why fibroblast data is presented.

Answer: Thank you for the comment. With regards to the comment about calcium retention in the siNEG/Dl cells, calcium FI only goes up around 20% compared to siNEG/Dl- cells, far less compared to siATP2A2/Dl. This data has been moved the extended view (**EV2**) as we have added new data on patient derived primary keratinocytes that are more pathophysiologically relevant. We acknowledge that there may have been a misunderstanding in the experimental setup due to lack of details on our end. To this regard, we have specified that in the si*ATP2A2* cell model we did not use acute Tg treatment as we did in the DDK and Tg-HEKa models and instead used acute Dl treatment. Finally, as suggested, we have added single cell calcium traces (**EV2 F-K**).

Given suggestion of therpeutic effect, have the authors considered RNA sequencing of normal keratinocytes, their atp2a2 knockdown cells, and the same cells treated with DL to show global normalization of gene function.

Answer: That is a good suggestion, however because of other reviewers' and the editor's request of primary patient keratinocytes, we had to focus our resubmission experiments there.

Overall, my greatest concern is the use of a non-physiologic model for DD. While the findings presented may be true, it is equally possible that they do not reproduce disease features of DD.

Answer: We acknowledge the reviewers preconceptions about the use of DD fibroblasts and as such have repeated many essential experiments in primary DDK keratinocytes (Figures 2-5).

4th Mar 2024

Dear Prof. Wikstrom,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now heard back from one referee who agreed to re-evaluate your manuscript. This referee also assessed your responses to concerns raised by referee #3. As you will see from the report below, the referee acknowledges the improvements of the revised manuscript but remains critical particularly regarding the calcium imaging and quantification. After a consultation with the referee and with my colleagues here, we agreed that raised concerns are justified and should be addressed in an additional and final round of major revision. We agreed that performing ratiometric measurements and calculations of calcium concentration are essential for further consideration of the manuscript. Other points incl. STIM1-gated ORAI channels and mitochondrial involvement should be addressed by discussion. Also, we agree with the referee that the manuscript would be better suited as a Report. Therefore, please check "Author Guidelines" and format your manuscript as a report.
https://www.embopress.org/page/journal/17574684/authorguide#reportsarticleguide

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- Rename "Conflict-of-interest statemen" to "Disclosure and competing interests statement". 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 policy https://www.embopress.org/competing-interests and update your competing interests if necessary.

Further consideration of a revision that addresses reviewer's concerns in full will entail an additional round of review. Acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

*****

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- 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.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

See detailed instructions here:

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11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

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- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

13) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

14) A Conflict of Interest statement should be provided in the main text.

15) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-800 px high.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors performed additional experiments and thus addressed some of the comments from the three reviewers. Nevertheless, some questions remain unanswered. These are described below and some are of essential importance for the claims of this paper.

Compared to other paper published in EMBO Mol Med the amount of data presented here is not on the higher end. Especially with the current figure organization (see for example Fig. 1 with only 3 panels) it would be, in my opinion, relatively easy to present the study as a report.

Regarding my comment about calcium imaging. I am somewhat puzzled as to why the authors used Fura Red and Rhod-2 for their cytosolic and mitochondrial measurements, respectively. These dyes do not allow ratiometric measurements and hence, calculation of the calcium concentration. My suggestion that normalizing to 1 is not appropriate for this study has been ignored. Without proper calibration of the calcium signals in all three compartments the claims of this paper are not fully supported by the data.

24 hours with Tg is more than sufficient to induce ER stress and Fig. 4B indicates that. I am not sure if I understand the explanation correctly. Do the authors suggest that ER stress has no functional role in this experimental system?

The new NFAT data indicate that indeed the cytosolic calcium is essential and obviously altered in Dl-treated cells and mutant DDK patient cells. This again indicates that proper measurements and precise calibration of intracellular calcium are essential for this study. The authors did not comment on the contribution of STIM1-gated ORAI channels. As mentioned in my first review, STIM1 is proposed as a biomarker of DD. Is dantrolene affecting their activity?

The GO term analysis indicated significant mitochondrial involvement. I understand the concern of the authors that analyzing mitochondrial function requires significant work. Nevertheless, to better understand DD development and therapy a more comprehensive evaluation of the whole cellular reprogramming is essential. Furthermore, mitochondria are known to control ER Ca2+ store depletion as well as ORAI channel function.

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Answer: We have now performed ratiometric measurements and calculations of calcium concentrations (Figure 1D-K).

Please also amend following points:
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1. Please note that the legends for figures 3i-j is not provided in the sequential manner (legend for figure 3j is provided before legend of figure 3i). This needs to be rectified.
Answer: We have corrected the sequential manner of call outs in the figure legends following reorganisation into a report.

2. Please define the annotated p value * in the legends of figures 3i, k; as appropriate.
Answer: We have corrected this for the now Figure 2I, K.

3. Please indicate the statistical test used for data analysis in the legends of figures EV 1b-c.
Answer: This has now been corrected.

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Answer: This has been corrected for the now Figure 1J and Figure 3A-D

5. Please note that information related to n is missing in the legends of figures 3i, k.
Answer: This has been updated for the new Figure 2I and 2K

6. Please note that the error bars are not defined in the legends of figures EV 2b-c; EV 3a-b.
Answer: We have added the error bar definitions to Figures EV3A-B

7. Please note that scale bar and its definition are missing for figure 1a.
Answer: Scale bar has been added to Figure 1A

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Answer: We have now combined all appendix figures into a single PDF titled ‘Appendix’, and referred to call-outs within the manuscript.

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Answer: This has been updated on the title page.

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Without proper calibration of the calcium signals in all three compartments the claims of this paper are not fully supported by the data.

Answer: That you for taking the time to review our manuscript again as well as your comment. With regards to this point, Fura Red is a ratiometric cytosolic calcium indicator, and so we have now performed additional experiments calculating the calcium concentration. As the mitochondrial calcium experiments were peripheral to the aim of the paper, as well as the fact that Rhod-2 is not ratiometric, we have not included that in the revised version, and have instead focussed on the Fura Red experiments.

24 hours with Tg is more than sufficient to induce ER stress and Fig. 4B indicates that. I am not sure if I understand the explanation correctly. Do the authors suggest that ER stress has no functional role in this experimental system?

Answer: Thanks for your comment. We treated normal primary keratinocytes (HEKa) with low levels of Tg for 24 hours in order to induce a pharmacological model of SERCA2 inhibition, in concert with the primary DD keratinocytes and si KD model. In the ER stress experiment that you refer to we used Tg pretreatment and then investigated whether dantrolene had a rescue effect.

The new NFAT data indicate that indeed the cytosolic calcium is essential and obviously altered in DI-treated cells and mutant DDK patient cells. This again indicates that proper measurements and precise calibration of intracellular calcium are essential for this study. The authors did not comment on the contribution of STIM1-gated ORAI channels. As

mentioned in my first review, STIM1 is proposed as a biomarker of DD. Is dantrolene affecting their activity?

Answer: Thank you for the comment. You raise a valid point. As we have reformatted as a report, we were not able to include further experiments looking into the role of STIM-gated ORAI channels, although we have however mentioned the potential importance in the relevant results/discussion section.

The GO term analysis indicated significant mitochondrial involvement. I understand the concern of the authors that analyzing mitochondrial function requires significant work. Nevertheless, to better understand DD development and therapy a more comprehensive evaluation of the whole cellular reprogramming is essential. Furthermore, mitochondria are known to control ER Ca2+ store depletion as well as ORAI channel function.

Answer: Again, this is another valid point, but unfortunately as we have reformatted this manuscript into a report, we have been unable to include additional experiments to investigate mitochondrial involvement. We have again however mentioned this in the relevant results/discussion section.

Dear Prof. Wikstrom,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now heard back from one referee who agreed to re-evaluate your manuscript. This referee also assessed your responses to concerns raised by referee #3. As you will see from the report below, the referee acknowledges the improvements of the revised manuscript but remains critical particularly regarding the calcium imaging and quantification.
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report. https://www.embopress.org/page/journal/17574684/authorguide#reportsarticleguide
Answer: It is formatted as a report.

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Answer: This has been updated for the new Figure 2I and 2K

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Answer: Scale bar has been added to Figure 1A

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and uploaded as such with the callout updated in the manuscript accordingly.
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Answer: This has been updated on the title page.

7th Mar 2024

Dear Prof. Wikstrom,

Thank you for your response to the editorial decision on your manuscript entitled "Dantrolene corrects cellular disease features of Darier disease and may be a novel treatment". I have now carefully examined the arguments provided in your letter and discussed them with the other members of our editorial team. Additionally, I have sought external advice on the study from an expert in the field.

I regret to inform you that we will not be able to reverse our original decision. We do acknowledge the improvements of the revised manuscript in particular the experiments with primary keratinocytes; however, in line with our editorial assessment our adviser agreed with the concern raised by the referee #1 regarding calcium imaging and quantification. Below are the comments from our adviser for your consideration.

- The reviewer asks to use ratiometric Ca2+ indicators, which is a valid request. The authors use FuraRed, which is indeed ratiometric, but do not convert the fluorescence value in [Ca2+]. This can be done by performing the usual calibration with cell permeabilization/Ca2+ supplementation/Ca2+ chelation. It is standard practice to do so. In my opinion, normalization of FuraRed shall be done.
- Along the same line, I don't understand how FuraRed was imaged. The details in the methods section are scant, with a reference to the in-built filters of the microscope, but common practice is to give details about the measurement because this dye is used in ratiometric measurements using excitation wavelengths of 420 nm and 480 nm or 457 nm and 488 nm. It is advisable that the authors specify that they used the 457-488 lasers and the bandwidth of the emission filter used to collect the images, as well as the procedure for ratioing and calculating the FuraRed ratio. These details appear to be pedantic but are essential to interpret the Ca2+ measurements carried out by the authors.
- The rhod2 indicator is non-ratiometric and has a high Kd, meaning that it is saturated at mito [Ca2+] of 1 microM or less, way below the actual Ca2+ concentrations reported to occur in mitochondria especially upon IP3-mediated Ca2+ signaling. The picture is however different when Ca2+ discharge from the ER is induced by SERCA inhibition with TG, as authors do here. Under these circumstances, mitochondrial Ca2+ uptake is much reduced and therefore Rhod2 might work. However, it is clear from the traces presented by the authors that Rhod2 is readily saturated also in their experimental conditions, as evidenced by the fact that DI and control treated cells reach the same peak in mitochondrial Ca2+ concentrations upon TG treatment. In any case, this experiment is peripheral to the message of the paper, unless authors want to say that less ER Ca2+ in DI treated cells leads to lower mitochondrial Ca2+ overload, which must be measured in completely different experiments.
- Did the authors pretreat cells with ionomycin before measuring Rhod2? This is unclear from the methods, and is clearly saturating the signal, so these measurements can be uninterpretable.
- Unclear if Ca2+ experiments were performed in Ca2+ free media (as advisable).
- These can all be corrected during revision as they are doable in 2 months. I would suggest that the authors go for another round of revision and perform additional Ca2+ experiments.

At this point, I would also like to clarify that we have invited all three initial referees to re-review your revised manuscript; however, only referee #1 accepted and was therefore asked to assess your responses to concerns of other referees, who declined to re-evaluate the manuscript.

Taking our adviser's comments in consideration and together with the referee re-evaluation and our editorial assessment we would like to reiterate our invitation of the second round of major revision. We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

PLease use this link to login to the manuscript system and submit your revision: https://embomolmed.msubmit.net/cgi-bin/main.plex

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

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9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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See detailed instructions here:

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11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

13) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

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15) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

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EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Dear Prof. Wikstrom,

Thank you for your response to the editorial decision on your manuscript entitled "Dantrolene corrects cellular disease features of Darier disease and may be a novel treatment". I have now carefully examined the arguments provided in your letter and discussed them with the other members of our editorial team. Additionally, I have sought external advice on the study from an expert in the field.

I regret to inform you that we will not be able to reverse our original decision. We do acknowledge the improvements of the revised manuscript in particular the experiments with primary keratinocytes; however, in line with our editorial assessment our adviser agreed with the concern raised by the referee #1 regarding calcium imaging and quantification. Below are the comments from our adviser for your consideration.

- The reviewer asks to use ratiometric Ca2+ indicators, which is a valid request. The authors use FuraRed, which is indeed ratiometric, but do not convert the fluorescence value in [Ca2+]. This can be done by performing the usual calibration with cell permeabilization/Ca2+ supplementation/Ca2+ chelation. It is standard practice to do so. In my opinion, normalization of FuraRed shall be done.
- Along the same line, I don't understand how FuraRed was imaged. The details in the methods section are scant, with a reference to the in-built filters of the microscope, but common practice is to give details about the measurement because this dye is used in ratiometric measurements using excitation wavelengths of 420 nm and 480 nm or 457 nm and 488 nm. It is advisable that the authors specify that they used the 457-488 lasers and the bandwidth of the emission filter used to collect the images, as well as the procedure for ratioing and calculating the FuraRed ratio. These details appear to be pedantic but are essential to interpret the Ca2+ measurements carried out by the authors.
Answer: Thank you for the helpful comment. We have now clarified the specific experimental conditions in which we performed the Fura Red imaging and calculations within the methods section.

- The rhod2 indicator is non-ratiometric and has a high Kd, meaning that it is saturated at mito [Ca2+] of 1 microM or less, way below the actual Ca2+ concentrations reported to occur in mitochondria especially upon IP3-mediated Ca2+ signaling. The picture is however different when Ca2+ discharge from the ER is induced by SERCA inhibition with TG, as authors do here. Under these circumstances, mitochondrial Ca2+ uptake is much reduced and therefore Rhod2 might work. However, it is clear from the

traces presented by the authors that Rhod2 is readily saturated also in their experimental conditions, as evidenced by the fact that DI and control treated cells reach the same peak in mitochondrial Ca2+ concentrations upon TG treatment. In any case, this experiment is peripheral to the message of the paper, unless authors want to say that less ER Ca2+ in DI treated cells leads to lower mitochondrial Ca2+ overload, which must be measured in completely different experiments.
- Did the authors pretreat cells with ionomycin before measuring Rhod2? This is unclear from the methods, and is clearly saturating the signal, so these measurements can be uninterpretable.
- Unclear if Ca2+ experiments were performed in Ca2+ free media (as advisable).
- These can all be corrected during revision as they are doable in 2 months. I would suggest that the authors go for another round of revision and perform additional Ca2+ experiments.

Answer: Your in-depth comments and advice are much appreciated. Taking on board your advice, we have now removed the Rhod-2 mitochondrial calcium experiments from our manuscript and instead discussed the potential role of mitochondrial calcium in the relevant results/discussion section.

4th Jul 2024

Dear Prof. Wikstrom,

Please find enclosed the final reports on your manuscript. We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Molecular Medicine.

Yours sincerely,
Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

Referee #4 (Remarks for Author):

I have been called to evaluate the Ca2+ experiments at the last round of revision. I made specific requests on the use of Rhod2 and on the calibration of FuraRed. Authors performed the Fura Red calibration and decided to eliminate the mitochondrial Ca2+ measurements using Rhod2 because of the intrinsic problems with that probe. They also clarified methods for Ca2+ measurements. I therefore believe that they have addressed my concerns, at least partially, and that therefore I have no other outstanding issues with the Ca2+ measurements part of the paper.

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Corresponding Author Name: Jakob D Wikstrom	
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Manuscript Number: EMM-2023-18120-V2	

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

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.

Materials

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	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Figure S3

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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	Materials and Methods
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
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?	Yes	Materials and Methods
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 and Figure Legends

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Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
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.	Yes	Materials and Methods
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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	