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Spatial and temporal control of mitochondrial H2O2 release in intact human cells

Michaela Hoehne, Lianne Jacobs, Kim Lapacz, Gaetano Calabrese, Lena Murschall, Teresa Marker, Harshita Kaul, Aleksandra Trifunovic, Bruce Morgan, Mark Fricker, Vsevolod Belousov, and Jan Riemer
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Corresponding author(s): Jan Riemer (jan.riemer@uni-koeln.de)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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 **28th Jul 2021**

Thank you for submitting your study entitled "Thioredoxin reductase controls the capacity of peroxiredoxins to limit mitochondrial H2O2 release" [EMBOJ-2021-109169]. The manuscript has been assessed by three reviewers, whose reports are enclosed below.

As you will see, the referees find your study potentially interesting. However, they also raise some points that need to be addressed before they can support publication here. In particular, referee #3 is concerned that the main conclusions are based on the use of a single cell line and requests you to quantify respiratory rates in cells grown under glucose and galactose.

Given the interest of your findings, I am pleased to invite submission of a manuscript revised as indicated in the reports attached herein. I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

Referee #1:

This manuscript describes the use of the new HyPer7 H_2O_2 sensor to study the release of H_2O_2 by mitochondria. Some data were presented on this issue in the paper from last year describing the development of the sensor, but the authors of the current work sought to further explore the blanket statement from the highlights of the previous work: "Being controlled by the Trx system, H_2O_2 does not diffuse out from mitochondria." The major conclusion from the current work is that if there is high peroxiredoxin activity in the cytosol, and if peroxiredoxins are being reduced effectively by thioredoxin reductase activity, then appreciable H_2O_2 levels will not be detected in the cytosol. However, if either of these conditions is not met, then H_2O_2 will diffuse out of mitochondria and be detected. This is a valuable observation that seems to be reasonably supported by the data. However, the message of the work is slightly undermined by the written presentation. The text appears to contradict itself at times. Since the authors are trying to resolve contradictions, this state of affairs is not ideal!

p. 3 In line 3 of the abstract, the authors may mean "implicated" rather than "implied."

p. 3 Third-to-last line of abstract, by "cell-to-cell differences" the authors presumably are referring to differences between cell types? Not very clear, since cell-to-cell variation in a single culture, differences between the same cells treated in different ways, and differences between cell types are all presented in the study.

p. 3 It may be an overstatement to say "our data indicate that H_2O_2 mediated signaling likely occurs..." since no data on signaling is presented. More accurate would be: "Our data suggest that H_2O_2-mediated signaling would need to occur..." More substantially (though this reviewer is a bit outside the fields of H_2O_2 sensing, mitochondria ROS generation, and H_2O_2 signaling), it seems logical that H_2O_2 would not itself have to diffuse into the nucleus (for example) for it to signal there. H_2O_2 would just have to modify a signaling factor that would then diffuse into the nucleus. As long as that factor would resist reversal of the signal in the cytosol on the way to the nucleus, signaling could still happen even if the signal was picked up near the mitochondria. This may be splitting hairs, but perhaps the authors could at least specify that signaling would need to be "initiated" close to mitochondria.

p. 5 The sentence "In addition, H_2O_2 release from isolated mitochondria or increased extracellular H_2O_2 has been reported" does not make sense to this reviewer.

p. 6 "...it also increases the levels of the cytosolic anti-oxidant thioredoxin system, which limits effective functioning of the cytosolic two-Cys peroxiredoxins which act redundantly." What are the authors trying to say here about the relationship between

thioredoxin and peroxiredoxins? Why should either the thioredoxin system or an increase in this system limit PRDX1/PRDX2 activity? Isn't the thioredoxin system required for PRDX1/PRDX2 activity? If the authors meant that the thioredoxin system is the rate limiting step in recycling peroxiredoxins, they should state that clearly.

p. 12 Perhaps say, "Our data are in apparent contradiction to a recent study..." since the apparent contradiction does seem to be resolved by the authors.

p. 12 Figure 6D is cited at the end of the sentence "Adaptation thereby not only involves proteomic changes... can take place on a time scale of minutes..." but Figure 6D does not illustrate anything about time scale. This is an important point, so the authors should make clear from which data they derive the conclusion that adaptation can happen rapidly.

p. 12 There are many poorly organized sentences (or poorly thought-out concepts) that make it difficult at times for the reader to follow the arguments. An example is, "This indicates that the reducing half reaction during H_2O_2 detoxification by peroxiredoxins may limit H_2O_2 handling, rather than the levels of peroxiredoxins." The authors seem to be trying to say something quite simple-- that the rate of reduction rather than the rate of oxidation is rate-limiting for peroxiredoxin turnover. Currently, the "levels of peroxiredoxins" appears in the sentence as the alternative to "H_2O_2 handling" rather than as the alternative to "H_2O_2 detoxification by peroxiredoxins."

p. 15 "Previous contradictory observations" seems to refer to a pre-existing debate in the literature (since both sides of the contradiction are called "previous"), but no references are given. If the authors are referring only to the particular 2020 paper, that should be made more clear.

p. 16 In general, the use of the word "limit" in this manuscript is imprecise and bordering on infuriating. The statement "diffusion of mitochondrial H_2O_2 in the cytosol is limited by PRDX1 and PRDX2" seems to contradict the previous statement that reduction of the peroxiredoxins is the rate-limiting step.

p. 16 typo: "Furthermore, when we deleted both PRDX together. The HyPer7..." Also, the semicolon on the same line should be deleted because it is grammatically incorrect.

Figure 1B: Is the amount of H_2O_2 added each time increased, or do the concentrations refer to the total amount of H_2O_2 added cumulatively? To avoid confusion, perhaps the amount of each bolus added should be indicated by adding a "+" symbol, i.e., + 2 mM H_2O_2. There is enough room to extend the figure downward to accommodate the +.

Also in Figure 4A it is not clear how much H_2O_2 is added each time.

Referee #2:

Using a combination of genetically encoded redox sensors (i.e., HyPer7, roGFP) and the H_2O_2 generators DAO, all targeted to different subcellular sites, the authors demonstrate that H_2O_2 leaking from mitochondrial sources (in response to hypoxia/reoxygenation events, culturing cells in glucose-deprived medium or by treating cell with mitochondrial respiratory chain inhibitors) is handled by the cytosolic thioredoxin reductase (TXNRD1) and peroxiredoxin (PRDX1, PRDX2) system at least in cells with impaired antioxidant capacity such as HEK293T cells. Their findings also imply the existence of an H_2O_2 channel in the IMM whose identity remains to be uncovered. In general, the study is well-performed and the findings are intriguing. Yet, I do have a couple of comments that the authors may wish to address.

Comments:

1) Since auranofin not only targets cytosolic TXNRD1 but also its mitochondrial counterpart (TXNRD2), one may wonder whether this might play a role here as well.
2) Since both TXNRD1 and TXNRD2 are selenoenzymes, it might be interesting to see whether culturing cells in the presence of nanomolar concentrations of sodium selenite may have an impact on H_2O_2 released from mitochondria.
3) Expression of peroxiredoxins does not report on their oxidation status, thus the authors may consider to study their oxidation status under some of the challenges applied in this study. At least, a thorough discussion on this would be appropriate.
4) As a control, would treatment of cells with (mito-targeted) superoxide/hydroperoxyl radical scavengers blunt HyPer7 oxidation?
5) The authors found that culturing cells in the presence of galactose caused changes in TXNRD1 expression. Would this also apply for enzymes of the glutathione pathway such as both subunits of the cysteine-glutamate-ligase? Would this explain why HEK293 cells behaved differently from HepG2 cells? A cross-talk between both redox systems has been repeatedly shown.
6) Did the authors consider a role of mitochondrial peroxiredoxins (PRDX3 and PRDX5) in preventing H_2O_2 leakage from mitochondria? Similarly, would knockout/knockdown of TXNRD1 or TXNRD2 increase HyPer7 signals? At least, these apparent issues should be discussed in-depth.

Minor:
- Page 6: Revise sentence "Instead, they indicated...."

Referee #3:

This manuscript nicely describes how thiol peroxidase/reductase systems control the release of H_2O_2 from mitochondria in a specific cell type (HEK293) comparing their results to a previous study in HeLa cells (and including a limited number of experiments also in HeLa). While they have a strong case to show for the effects of thiol peroxidase/reductase systems in the control of mitochondrial H_2O_2 release, I don´t think they can generalize this to all cell types and conditions, as they do in the text.

Major points:

1. The use of a single cell line is complicated give the vast documented differences between cell types, cell lines and redox state. Conclusions made are very general considering only HEK293 and some confirmations of prior data in HeLa cells are shown. The authors must remember that, while their methodology is more elegant, there is a plethora of literature in different cells and tissues showing significant release of H_2O_2 from mitochondria without removing these antioxidant systems, and many of these may indicate tissue-specific characteristics of mitochondrial redox signaling.

2. While the HyPer7 system is very elegant and removes artifacts from pH, it is not very sensitive, as clearly indicated by the authors data showing responses to micromolar H_2O_2, and the small, albeit significant, effect of antimycin, which in most tissues is a strong inducer of electron leak. Thus, it may miss release at concentrations lower than its sensitivity (which may also coincide with affinity of the antioxidant systems studied). This should at least be acknowledged, since low H_2O_2 concentrations under the sensitivity of HyPer7 are still expected to have signaling effects.

3. Proof of concept that the detection sensitivity is low is that there often is no clear detection under basal conditions in any compartment.

4. Also missing is a discussion of possible differences in detection/sensitivity in the different target locations. I find no other plausible explanation for the larger effect of antimycin on the OMM versus IMM, for example.

5. Perhydroxyl radical formation can allow for superoxide radical re-entry into the matrix from the IMM - this possibility was not commented on.

6. Studying the difference between glucose and galactose as substrates is of interest, since cell lines are often highly glycolytic. However, we do not know how this cell line behaves in terms of energy metabolism. It would be important to quantify respiratory rates in cells grown under both conditions and compare to HeLa and any other cell type evaluated.

7. Only antioxidant proteins were quantified, but clearly the electron donor molecules are essential. NADPH, thioredoxin and glutathione should be measured as well.

Minor:

1. Auroforin and what it does should be explained when first used.

2. Complex III does not generate superoxide exclusively toward the IMM, although it is usually the most quantitatively relevant leakage localtion.

3. Antimycin A is also expected to increase electron leakage at complex I, as it blocks electron flow through I-III.

4. Why wasn´t rotenone used, since the HeLa paper used it?

POINT-BY-POINT RESPONSE

Referee #1:

p. 3 In line 3 of the abstract, the authors may mean "implicated" rather than "implied."
We now write “implicated”.

p. 3 Third-to-last line of abstract, by "cell-to-cell differences" the authors presumably are referring to differences between cell types? Not very clear, since cell-to-cell variation in a single culture, differences between the same cells treated in different ways, and differences between cell types are all presented in the study.
We were referring to differences between different cell types. We now replaced “cell-to-cell” differences by “differences between cell types”.

p. 3 It may be an overstatement to say "our data indicate that H2O2 mediated signaling likely occurs..." since no data on signaling is presented. More accurate would be: "Our data suggest that H2O2-mediated signaling would need to occur..." More substantially (though this reviewer is a bit outside the fields of H2O2 sensing, mitochondria ROS generation, and H2O2 signaling), it seems logical that H2O2 would not itself have to diffuse into the nucleus (for example) for it to signal there. H2O2 would just have to modify a signaling factor that would then diffuse into the nucleus. As long as that factor would resist reversal of the signal in the cytosol on the way to the nucleus, signaling could still happen even if the signal was picked up near the mitochondria. This may be splitting hairs, but perhaps the authors could at least specify that signaling would need to be "initiated" close to mitochondria.
We now write " Our data suggest that H_2O_2-mediated signaling is only initiated in close proximity to mitochondria during specific metabolic conditions. ".

p. 5 The sentence "In addition, H2O2 release from isolated mitochondria or increased extracellular H2O2 has been reported" does not make sense to this reviewer.
Measuring H_2O_2 release from isolated mitochondria or measuring extracellular H_2O_2 was in the past used as proxies for H_2O_2 release from mitochondria in intact cells. We now rephrased the sentence to: “In addition, H_2O_2 release was assessed using isolated mitochondria or measurements of extracellular H_2O_2 (both as proxies for mitochondrial H_2O_2 release inside cells)”

p. 6 "...it also increases the levels of the cytosolic anti-oxidant thioredoxin system, which limits effective functioning of the cytosolic two-Cys peroxiredoxins which act redundantly." What are the authors trying to say here about the relationship between thioredoxin and peroxiredoxins? Why should either the thioredoxin system or an increase in this system limit PRDX1/PRDX2 activity? Isn't the thioredoxin system required for PRDX1/PRDX2 activity? If the authors meant that the thioredoxin system is the rate limiting step in recycling peroxiredoxins, they should state that clearly.
We have re-written this section to improve clarity. The reviewer is of course completely correct that the reduction of peroxiredoxins, which is typically mediated by thioredoxins is the rate limiting step in peroxiredoxin-catalyzed H_2O_2 reduction. However, this is not what we meant in this section or in the manuscript in general. Rather we claim that the rate-limiting reduction of peroxiredoxins by the thioredoxin system is itself rate-limited and controlled by thioredoxin reductase availability. Differences in thioredoxin reductase level are observed between cells grown with glucose and galactose as carbon source and between HEK293 and HeLa cells. In both cases the change in thioredoxin reductase level correlates well with the cytosolic H_2O_2 scavenging capacity as inferred by the detection of mitochondrial H_2O_2 in the cytosol and by the monitoring HyPer7 probes in defined intracellular locations in response to exogenous H_2O_2. For clarity, and as discussed in the discussion

section, we believe that the lack of detection of mitochondrial H_2O_2 in the cytosol does not mean that no mitochondrial H_2O_2 is released into the cytosol. On the contrary, we claim (consistent with other recent studies) that the cytosolic H_2O_2 scavenging capacity in these situations is so efficient that there is a very steep H_2O_2 concentration gradient emanating from the mitochondria, which is not possible to detect with a HyPer7 probe that is freely soluble in the cytosol and that report on the average H_2O_2 level of the whole cytosol.

p. 12 Perhaps say, "Our data are in apparent contradiction to a recent study..." since the apparent contradiction does seem to be resolved by the authors.
We included the word “apparent”

p. 12 Figure 6D is cited at the end of the sentence "Adaptation thereby not only involves proteomic changes... can take place on a time scale of minutes..." but Figure 6D does not illustrate anything about time scale. This is an important point, so the authors should make clear from which data they derive the conclusion that adaptation can happen rapidly.
We thank the reviewer for raising this important point. We were referring to a data set that we had not included in the previous version of the manuscript. It is now included as **novel Figure S5**. Here, we shifted cells from glucose to galactose for 30 min (no long-term adaptation) and the response to mtDAO-derived H_2O_2 was monitored. Already after such a short time, the HyPer7 response was attenuated consistent with a rerouting of carbohydrate metabolism to provide more NADPH.

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We thank the referee for pointing out poor language and difficult-to-understand sentences/paragraphs in our manuscript. Together with the native speakers who co-author this study we now carefully reworked the entire text.

p. 15 "Previous contradictory observations" seems to refer to a pre-existing debate in the literature (since both sides of the contradiction are called "previous"), but no references are given. If the authors are referring only to the particular 2020 paper, that should be made more clear.
We now cited Pak et al, 2020 at this position.

p. 16 typo: "Furthermore, when we deleted both PRDX together. The HyPer7..." Also, the semicolon on the same line should be deleted because it is grammatically incorrect.
We corrected the typo and removed the semicolon.

Figure 1B: Is the amount of H2O2 added each time increased, or do the concentrations refer to the total amount of H2O2 added cumulatively? To avoid confusion, perhaps the amount of each bolus added

should be indicated by adding a "+" symbol, i.e., + 2 mM H2O2. There is enough room to extend the figure downward to accommodate the +.

The amounts of H_2O_2 indicated in **Figure 1B** show the amount applied each time. We added the "+" as suggested by the referee.

Also in Figure 4A it is not clear how much H2O2 is added each time.

The amounts added are 2, 4 and 8 μM, respectively. We added this information to the figure legend and the figure.

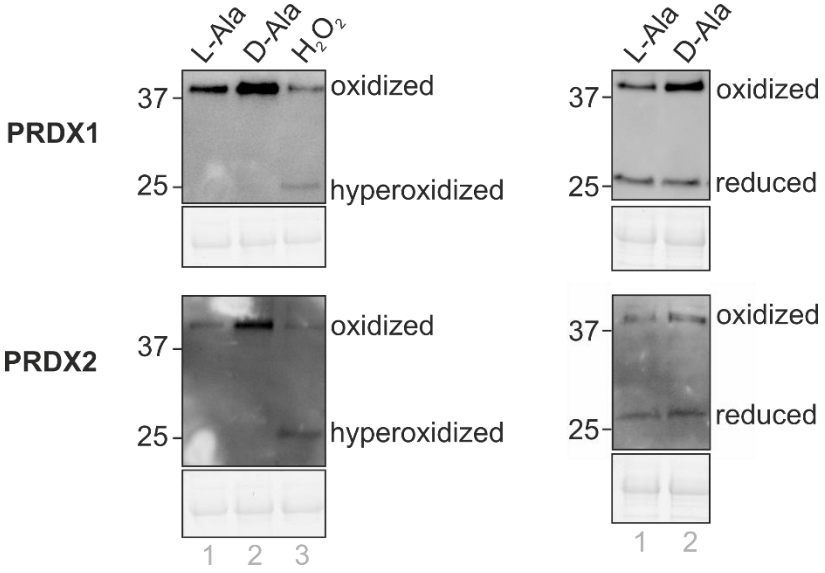
Referee #2:

1) Since auranofin not only targets cytosolic TXNRD1 but also its mitochondrial counterpart (TXNRD2), one may wonder whether this might play a role here as well.
2) Since both TXNRD1 and TXNRD2 are selenoenzymes, it might be interesting to see whether culturing cells in the presence of nanomolar concentrations of sodium selenite may have an impact on H2O2 released from mitochondria.

In our analysis of matrix H_2O_2 dynamics using the HyPer7 and roGFP2-scPrx1 sensors (**Figure 5D,E**), we did not observe an increase in response of the sensors upon treatment with auranofin (as we did for their cytosolic counterparts). To the contrary, the HyPer7 sensor revealed an attenuated response upon auranofin treatment. Our data on the dynamics of the glutathione pool (see below) indicate that in the matrix, glutathione reductase might play an important role in H_2O_2 handling as BCNU treatment resulted in a strong sensitization of the matrix HyPer7 sensor. Notably TXNRD2 levels are not affected by growth on galactose (**Figure 5A**).

3) Expression of peroxiredoxins does not report on their oxidation status, thus the authors may consider to study their oxidation status under some of the challenges applied in this study. At least, a thorough discussion on this would be appropriate.

This is a good point. Using a protocol by Cox et al (Methods in Enzymology, Vol 474, 2010), we analysed, in HEK293 cells, the interconversion of PRDX1 and PRDX2 between their three main redox forms: reduced, oxidized, and hyperoxidized during selected treatment conditions. When we compared D-Ala with L-Ala-treated cells (4 mM), we found that oxidation but not hyperoxidation of PRDX1 and PRDX2 increased. Upon treatment with a high amount of external H_2O_2 (200 µM = 20 times more that we normally use throughout the manuscript), we did observe an increase in hyperoxidized PRDX1 and PRDX2. This is in line with previous results (in Jurkat cells, PRDX1 and PRDX2 became hyperoxidized between 20 and 100 µM external H_2O_2). Thus, we think that under the conditions employed in our study, PRDX1 and PRDX2 become oxidized as part of their activity cycle but do not become hyperoxidized.



Interconversion of PRDX1 and PRDX2 redox states in HEK293 cells treated with H_2O_2 or with D-Ala to induce mtDAO-dependent H_2O_2 production. We utilized two assays developed by Cox et al to analyse on the one hand the difference between hyperoxidized (potentially inactivated) and non-hyperoxidized PRDXs and on the other hand the difference between reduced and oxidized PRDX. With the selected treatments that are more severe than the ones used throughout our study, we observe that PRDX1 and PRDX2 become oxidized but hardly hyperoxidized. We thus assume that both PRDXs stay fully active throughout all treatments employed in our study.

4) As a control, would treatment of cells with (mito-targeted) superoxide/hydroperoxyl radical scavengers blunt HyPer7 oxidation?

In the course of this revision, we performed experiments in which we combined antimycin A and mito-Q treatment. Notably, in these experiments, we did not observe differences of mito-HyPer7 responses upon antimycin A treatment. At present, we do not know why this is, but we plan to explore HyPer7 responses during such treatments in future studies.

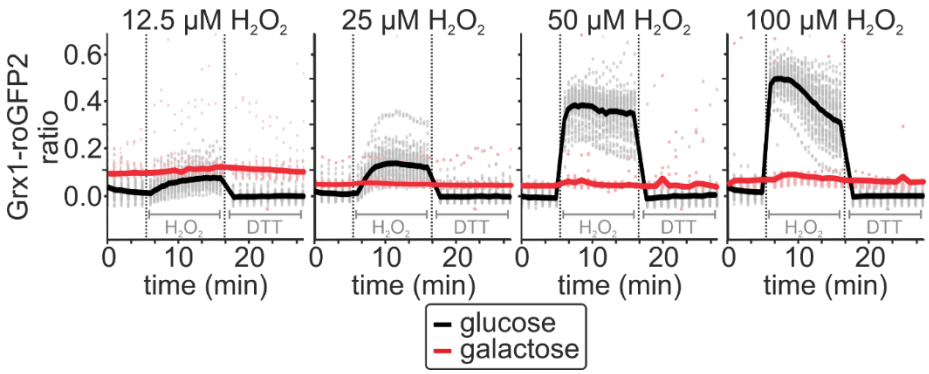
5) The authors found that culturing cells in the presence of galactose caused changes in TXNRD1 expression. Would this also apply for enzymes of the glutathione pathway such as both subunits of the cysteine-glutamate-ligase? Would this explain why HEK293 cells behaved differently from HepG2 cells? A cross-talk between both redox systems has been repeatedly shown.

These are very good points raised by the referee (we assume the referee means "HeLa" and not "HepG2" cells). We performed different experiments to address the impact of the glutathione system. We provide these data for the referees only and do not include them in the manuscript as we feel that the manuscript already contains a lot of data and would become overloaded with the risk of the important messages being obscured if we would include these data. However, if the reviewers consider it important we are happy to add them upon request.

We first checked our proteomics data and found glutathione synthase and the regulatory unit of glutamate-cysteine-ligase to be unchanged between glucose and galactose-grown cells. The catalytic unit of glutamate-cysteine ligase was changed with a slight increase by 1.33 on galactose compared to glucose (-log p = 1.79). Since this enzyme catalyzes the rate-limiting step of glutathione biosynthesis, it might be that this positively impacts the availability of glutathione.

We then investigated how glutathione dynamics (E_{GSH}) crosstalk with compartmental H_2O_2 handling using the glutathione sensor Grx1-roGFP2 (Gutscher et al, Nature Methods 2008; employed by us: Fischer et al, MBoC 2013, Kojer et al, EMBO J 2012, Calabrese et al, EMBO J 2019).

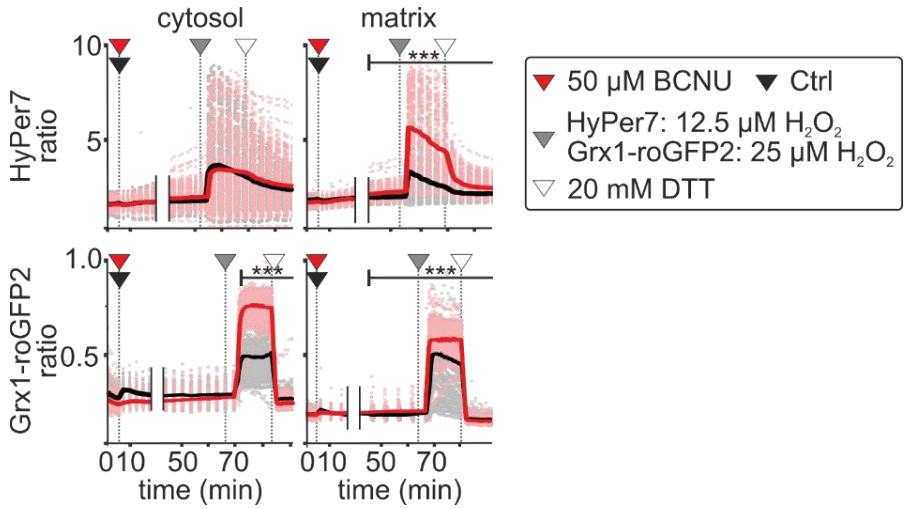
We first asked whether E_{GSH} dynamics upon exogenous H_2O_2 addition was changed in cells grown in galactose compared to glucose. We found cytosolic E_{GSH} to be much more robust against exogenous H_2O_2 when cells were grown on galactose. This indicates that the improved H_2O_2 handling in galactose-grown cells directly impacts E_{GSH} dynamics.



Response of a Grx1-roGFP2 probe in the cytosol of HEK293 cells to external application of H_2O_2. HEK293 cells were grown either with glucose (black curve and data points) or galactose (red curve and data points) as carbon source. Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. Grx1-roGFP2 responses are less sensitive in cells grown on galactose.

We also tested how differences in E_{GSH} dynamics affected H_2O_2 handling. To this end, we inhibited mitochondrial and cytosolic glutathione reductase by BCNU. H_2O_2 addition impacted E_{GSH} upon H_2O_2 incubation in both compartments although cytosolic E_{GSH} appeared to react even more sensitive. By

comparison, the HyPer7 response was only changed in the matrix but not in the cytosol. Thus, in mitochondria there appears to be a relevant crosstalk between the glutathione system and H_2O_2 handling. This might influence mitochondrial H_2O_2 handling and thus the amount of H_2O_2 available for release. Conversely, cytosolic glutathione reductase does not contribute to H_2O_2 treatment suggesting that cytosolic E_{GSH} does not influence mitochondrial H_2O_2 release.



Response of HyPer7 and Grx1-roGFP2 probes in the cytosol and matrix of HEK293 cells to external application of H_2O_2 after treatment of cells with the glutathione reductase inhibitor BCNU. HEK293 cells were treated either with BCNU (red curve and data points) or ethanol as control (black curve and data points). After 60 min incubation time, the response of the sensors towards external H_2O_2 was tested. Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. BCNU affects glutathione handling in cytosol and matrix (to a lesser extent in the matrix) but only affects HyPer7 deflection in the matrix indicating that in the cytosol glutathione impacts less on H_2O_2 handling.

In summary, we consider that our data suggest that changes in glutathione homeostasis are of minor importance, if at all, for our findings.

6) Did the authors consider a role of mitochondrial peroxiredoxins (PRDX3 and PRDX5) in preventing H2O2 leakage from mitochondria? Similarly, would knockout/knockdown of TXNRD1 or TXNRD2 increase HyPer7 signals? At least, these apparent issues should be discussed in-depth.

Mitochondrial PRDX3 and PRDX5 influence matrix H_2O_2 handling and thus very likely also the levels of H_2O_2 that can be released from mitochondria. However, mitochondrial antioxidative systems do not appear to be changed between cells grown on galactose or glucose. In line with this, H_2O_2 amounts in the matrix upon mtDAO-based H_2O_2 generation are similar between glucose and galactose (Figure 3C), and thus differences in cytosolic H_2O_2 under these conditions cannot be linked to PRDX3 and PRDX5.
In our opinion, knockout of TXNRD1/2 would affect cells similarly as acute treatment with auranofin (naturally, knockouts might result in unforeseeable adaptation effects)

Minor:
- Page 6: Revise sentence "Instead, they indicated...."

We revised the sentence and added additional context. It now reads as follows: “Notably, experiments with HyPer7 cast doubt on the release of H_2O_2 from mitochondria towards the cytosol (Pak et al., 2020). No H_2O_2 release could be detected upon rotenone- or matrix-targeted D-amino acid oxidase-induced mitochondrial H_2O_2 generation. Only upon inhibition of the cytosolic thioredoxin system, could mitochondrial H_2O_2 diffuse into the cytosol at a measurable amount.”

Referee #3:

1. The use of a single cell line is complicated give the vast documented differences between cell types, cell lines and redox state. Conclusions made are very general considering only HEK293 and some confirmations of prior data in HeLa cells are shown. The authors must remember that, while their methodology is more elegant, there is a plethora of literature in different cells and tissues showing significant release of H2O2 from mitochondria without removing these antioxidant systems, and many of these may indicate tissue-specific characteristics of mitochondrial redox signaling.

We agree with this referee that the use of a single cell line (HEK293 cells) limits our study. While it is beyond the scope of our study to provide all data with a panel of different cell lines, we did perform an additional experiment in which we assessed antimycin A-dependent H_2O_2 release from mitochondria in HEK293, HeLa, 143B and COS-7 cells (**novel Figure S5**). In all cell lines, we observed deflection of the HyPer7 sensor in the matrix. Conversely, only in HEK293 and COS-7 cells, we observed HyPer7 deflection in the cytosol compared to the control. In HeLa and 143B cells no HyPer7 deflection could be observed supporting the claim that different cell types have different capacities to efficiently scavenge H_2O_2 released from mitochondria.

2. While the HyPer7 system is very elegant and removes artifacts from pH, it is not very sensitive, as clearly indicated by the authors data showing responses to micromolar H2O2, and the small, albeit significant, effect of antimycin, which in most tissues is a strong inducer of electron leak. Thus, it may miss release at concentrations lower than its sensitivity (which may also coincide with affinity of the antioxidant systems studied). This should at least be acknowledged, since low H2O2 concentrations under the sensitivity of HyPer7 are still expected to have signaling effects.

3. Proof of concept that the detection sensitivity is low is that there often is no clear detection under basal conditions in any compartment.

We agree with this referee that an even more sensitive H_2O_2 probe would be desirable and we now acknowledge the shortcomings of HyPer7 when introducing the probe. However, we think for the following reasons that HyPer7 sensitivity might be (close to) sufficient to monitor real-time changes in endogenous H_2O_2 dynamics:

1/ intramolecular H_2O_2 levels have been estimated to be in the range of 1-100 nM (Sies and Jones, Nature Rev Mol Cell Biol 2020). HyPer7 is capable of monitoring nanomolar amounts of H_2O_2. because low µM amounts of external H_2O_2 translate to nanomolar amounts of intracellular H_2O_2.

2/ Whenever we monitored <u>untreated</u> cells, we consistently observed that HyPer7 became progressively more reduced during the course of the measurement. This is reminiscent of what has been observed in yeast cells for the roGFP2-Tsa2 sensor (Morgan et al, Nature Chem Biol 2016). In the case of this sensor the decrease in probe signal was correlated to decreasing H_2O_2 generation due to decreasing endogenous oxygen levels during the measurements. This might also be the case for our HyPer7 measurements as we exchanged medium before the experiments presumably resulting in increased endogenous oxygen levels that then decrease with time.

3/ while in wildtype cells, HyPer7 at steady state is almost completely reduced because DTT did not reduce it any further, in PRDX1/PRDX2 knockout cell HyPer7 at steady state is more oxidized and could be further reduced by DTT addition (**novel Figures S1**). This indicates that the sensitivity of HyPEr7 is sufficient to monitor endogenous H_2O_2 levels e.g. upon genetic or pharmacological disturbances.

4. Also missing is a discussion of possible differences in detection/sensitivity in the different target locations. I find no other plausible explanation for the larger effect of antimycin on the OMM versus IMM, for example.

This is an excellent point, and we now discuss this shortly in the manuscript. Although we can only hypothesize, we think that especially differences in the reducing half reaction of the HyPer7 sensor (*i.e.* concentrations of thioredoxins, accessibility of HyPer7 for thioredoxins) contribute to the apparent differences in HyPer7 sensitivity on different compartments.

5. Perhydroxyl radical formation can allow for superoxide radical re-entry into the matrix from the IMM - this possibility was not commented on.

We included this in the manuscript text at the position where we speculate how matrix HyPer7 might be deflected by antimycin A-induced H_2O_2 release (Figure 1E, p8).

6. Studying the difference between glucose and galactose as substrates is of interest, since cell lines are often highly glycolytic. However, we do not know how this cell line behaves in terms of energy metabolism. It would be important to quantify respiratory rates in cells grown under both conditions and compare to HeLa and any other cell type evaluated.

This is a good point and we now performed oxygen consumption assays with HEK293 cells grown on glucose and galactose (**novel Figure S2**). As probably expected, we thereby found that HEK293 cells grown on galactose have a slightly higher basal oxygen consumption rates (OCR) and lower extracellular acidification rates (ECAR) compared to glucose grown cells.

This is in line with data reported for many other cell lines (glucose vs galactose growth), e.g. L6 cells (skeletal muscle cell line) [Dott et al, Redox Biol 2014], RPE1 cells (retinal pigment epithelial cell line) [Macvicar et al, J. Cell Sci 2014], fibroblasts [Gohil et al, Nature Biotechnology 2010] or HCT116 cells (a colorectal carcinoma cell line) [Weinberg et al, PNAS 2010].

7. Only antioxidant proteins were quantified, but clearly the electron donor molecules are essential. NADPH, thioredoxin and glutathione should be measured as well.

We provide additional data on E_{GSH} reflecting the dynamics of the glutathione pool (**see answer to comment from referee #2**). We also tried to provide data on NADPH – unfortunately the relatively novel genetically encoded NADPH sensors, iNap1 and iNap2, gave no reliable data in our hands. Thioredoxin 1 and 2 protein levels did not change in proteomics.

Minor:

1. Auroforin and what it does should be explained when first used.

We introduced auranofin already in the previous version of manuscript as “inhibited thioredoxin reductase using auranofin (Figure 5C)” (**Figure 5C**, p.11)”

2. Complex III does not generate superoxide exclusively toward the IMM, although it is usually the most quantitatively relevant leakage location.

We mentioned this already in the previous version of the manuscript but emphasized it now by including the word “mainly”: “Complex I and III release ROS to different sides of the mitochondrial inner membrane (IMM), complex I towards the matrix and complex III mainly towards the intermembrane space (IMS) (Brand, 2010, Han, Williams et al., 2001) (although release of ROS from complex III to the matrix has also been reported (Muller, Liu et al., 2004)).” (p.4)

3. Antimycin A is also expected to increase electron leakage at complex I, as it blocks electron flow through I-III.

We emphasize this now when introducing the use of antimycin A by adding the following sentence: “but likely also increases superoxide production at complex I” (p.8)

4. Why wasn´t rotenone used, since the HeLa paper used it?

We also performed the experiment with rotenone and included it as **novel Figure S3**. In this setting, we observed a lower deflection of the HyPer7 sensor in the cytosol particularly when using HEK293 cells grown on glucose. In galactose-grown HEK293 cells, a deflection of cyto-HyPer7 is observable.

1st Revision - Editorial Decision **4th Jan 2022**

Thank you for submitting the revised version of your manuscript. As you may know, Elisabetta Argenzio left The EMBO Journal last year. Before doing so, she transferred your manuscript to me as handling editor and I sent it back to the three initial referees from which we have now received comments (please see below). Referee #1 and #2 find that their comments have been satisfactorily addressed and now explicitly support publication. As referee #3 also does not raise any further specific issues, we have decided to proceed with the study. Therefore, I would now ask you to address a number of editorial issues that are listed in detail below in a final revised version of the manuscript. Please add all final edits using the "track changes" option in the manuscript file the data editors have added their notes to (please see below). Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Referee #1:

The authors have made efforts to improve the clarity of manuscript based on this reviewer's criticisms. They also show additional experiments and provide additional discussion relating to the other reviewers' comments. They make a convincing case that thioredoxin reductase levels differ depending on the metabolic state of the cell (or the cell type), which in turn affects the ability of cytosolic peroxiredoxins to eliminate hydrogen peroxide derived from mitochondrial activity.

Referee #2:

The authors have appropriately addressed all of my concerns and performed two more important experiments. Since I deem both sets of experiments important and relevant (the second exp. was also asked for by Reviewer 3), I suggest to include both of them in the revised version. Beyond this, I have no further comments.

Referee #3:

While the authors have attempted to respond to reviewer comments, the manuscript´s main weakness persists. It still has little novelty, as its main findings are compatible with a plethora of papers already published. It also is mostly focused on one cell type (while acknowledging there are substantial differences in other cell types) and cell growth conditions (mostly high glucose, high oxygen) that differ widely from in situ situations. In all, while there is not much wrong with the paper in its current form, there is also no great finding that would justify its publication in The EMBO Journal.

The authors have made all requested editorial changes.

Accepted **24th Jan 2022**

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jan Riemer
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109169

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.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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?	experiments were performed at least in three biological replicates, the n numbers are indicated in the Figure legends or Table S6 for each experiment. We did not use any statistical test to predeterminate the sample size. The sample size was chosen on the basis of our experience and good laboratory practice
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?	no data were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	all data were included; no groups/sets were preformed
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes, we present data with the Standard Deviation (SD).
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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

http://datadryad.org

http://figshare.com

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

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http://biomodels.net/miriam/
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	we provide this information in a table in the supplementary information, including catalogue number and supplier
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	source of cell lines is provided in the supplementary information

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

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	Data availability section is included
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA