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Glutathione redox potential in the mitochondrial intermembrane space is linked to the cytosol and impacts the Mia40 redox state

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1st Editorial Decision 10 February 2012

Thank you for the submission of your manuscript to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below. Although referee #3 is less positive towards the general interest of your study, the remaining two referees strongly support your manuscript. I would therefore like to ask you to revise it according to the referees' comments.

I believe that the referee reports are quite explicit and I will not repeat their concerns here. I would like, however, to point out that the suggestion of referee #2 regarding the analysis of the contribution of the respiratory chain and ROS, which has been corroborated by referee #1 in a private communication, needs to be attended to.

Please be aware that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

We generally allow three months as standard revision time. As a matter of policy, competing

manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS :

Referee #1:

The manuscript by Kojer et al. provides a comprehensive analysis of the glutathione redox dynamics in yeast using a redox sensor. The experiments are - as far as I can judge as a mammalian person - competently performed and analysed, warranting publication of the manuscript after a few issues as outlined below have been attended to.

Major issues:
The panels of Figure 1 should be labelled in the order of mention in the text, which is at present A, B, C, D, H, E, F, G, I, J. Figure 1G does not indicate the time points of the individual panels.
Legend to Figure 3A: Define Zwf1.
As Figures S2 and S3 are not mentioned in the manuscript, they can be deleted.
Figure 7 needs a short explanatory legend.

Minor issues:
The spelling should use consistently English or American spelling (according to Journal policy), at present we have both, "analyzed" and "analysed".
p. 3, line 19: delete "redox"
In my opinion, the statement at the bottom of p. 6 should be qualified (e. g., "did not significantly affect...") as the 30 min treatment with diamide potentially shows a very weak growth inhibition with the two highest dilutions (Figure S1).
Legend to Figure 2: The abbreviations Pgk1, Act1, Mrp1 and Mia40 need to be defined.
Figure S4: SOD and CCP1 need to be defined.
Figure 6: Define Erv1
p. 11, line 8 from bottom: italicise "N" in N-ethylmaleimide

Referee #2:

This manuscript is an important contribution to understand the fundamental phenomenon of the dynamic exchange of glutathione between the cellular compartments. The levels and oxidation status of glutathione is a major determinant of the redox environment in the cellular comportments, including the cytosol and mitochondria. Thus, the results of this study are critical for deciphering the capacity and interrelation of the redox-dependent processes and pathways.
The attempts to describe the redox milieu of the cell have been undertaken earlier. In this report, the authors utilized a special sensor constituting of the redox-sensitive GFP fused to glutaredoxin. According to the authors, this sensor has several advantages over the sensors used in the previous studies. The sensor was targeted to the cytosol, intermembrane space and the matrix to assess the levels of glutathione oxidation, redox potential, and to measure an ability of the system to rebuilt proper GSH:GSSG ratio after an oxidation stress due to the diamide treatment. The authors demonstrated that the pools of glutathione in the intermembrane space of mitochondria and the

cytosol equilibrated readily via porins. In contrast, the exchange of glutathione through the inner membrane between the intermembrane space and matrix is very inefficient (despite an obvious must for glutathione transporter in this membrane). Thus, the cytosol and the intermembrane space formed a non-interrupted milieu, and the glutathione redox status is maintained by the cytosolic glutathione reductase system therein.

The results presented in this study are of excellent quality. The authors argumentation and conclusions are convincing. The manuscript is well written, interesting and provides important data required to better understand the network of the redox pathways and processes operating in the cell. The "mechanistic" picture will be deepen by addressing the following mitochondria-related issues:

- The influence of the respiratory chain and reactive oxygen species on the glutathione redox buffer remains of high importance. The authors only touched this issue in the course of their analysis in the cells treated with the inhibitors antimycin A, KCN or paraquat. However, these measurements seem to be done after growth under fermentative conditions characterized by little or no respiration. To really conclude about the effects of the inhibitors, the authors should perform the measurements under respiratory conditions. Also, it would be interesting to compare the glutathione redox potentials of the cells lacking mitochondrial DNA (no respiration, no respiratory chain complexes). These additions would lead to better understanding the role of the respiratory complexes in redox homeostasis.

- The authors demonstrate that glutathione, in addition to the sulfhydryl oxidase Erv1, contributes to the redox state of MIA40 required for the transport and assembly of intermembrane space proteins. The role of Erv1 in maintaining the redox state of Mia40 is well known, and indeed confirmed by the authors in the current manuscript in the intact cells. What is the glutathione redox potential in the strains with depletion or overexpression of Erv1? Furthermore, the redox state of Mia40 has been demonstrated earlier to be dependent on the mutations in the respiratory chain complexes due to the fact that Erv1 passes the electrons derived from the Mia40 oxidation into cytochrome c and further to complex IV and molecular oxygen. So, in general complex III mutants have been shown to have more oxidized Mia40, whereas mutants in cytochrome oxidase (IV) rather opposite. It would be interesting to recapitulate these effects in vivo and also to measure the glutathione redox potential for mutant representatives, which were previously reported to affect redox state of Mia40.

- It would be very helpful to construct a table, in which the various measurements of the redox potentials for the cellular compartments are presented, including the data obtained in this study.

Minor:
- What is SS on the Figure 6B and 6D? Figure 6D: there is no sample without Dia, however the legend says otherwise.

Referee #3:

The authors are experts in redox biology and recently have developed suitable tools to measure intracellular redox levels in dynamically changing environments. These ratiometric tools are now applied to measure the redox level of the mitochondrial intermembrane space in comparison to the cytosol. This has been done earlier, and in one work (JBC) surprisingly a difference in redox potentials has been reported between these two compartments, despite the fact that it is known since the mid 1970s that small molecules exchange freely through the porins (and TOM) of the mitochondrial outer membrane. Since GSH is the major cellular redox buffer it is expected that it freely and rapidly exchanges between IMS and cytosol. This should lead to redox equilibrium and communication between cytosol and IMS. The knowledge of the redox status of the intermembrane space is of certain importance, because numerous proteins of this compartment are imported in a redox-dependent fashion via the well-studied MIA40 pathway. The authors now convincingly report that the redox potentials of the two compartments are similar and dynamically communicate via porin. This is documented by the finding that the cytosolic GSH reductase determines the redox potential also in the IMS. This situation is different for the mitochondrial matrix serving as a suitable control. The work has been performed at high standards and the results are compelling. The authors explain the differences to an earlier study by the use of different tools to measure the redox

levels, a reasonable suggestion. Overall, a nice study but the expected nature of the findings limits the impact of the study and hence it seems more suitable for a specialized biochemical journal.
Comments:

1. Surprisingly the large TOM pore cannot compensate for the porin deletion in GSH equilibration. The authors did not discuss why TOM does not appreciably compensate for the lack of porin in GSH trafficking as this has been documented earlier for other small molecules.
2. The authors should mention the pioneering work of Hackenbrock from the 70s and early 90s on the ionic strength similarities of the intermembrane space and the cytosol.

1st Revision - Authors' Response 08 May 2012

Point-by-point response to the comments of the referees

Referee #1

#1/ *The panels of Figure 1 should be labeled in the order of mention in the text, which is at present A, B, C, D, H, E, F, G, I, J. Figure 1G does not indicate the time points of the individual panels.*

A novel Figure S1 has been added. In the depicted experiment we titrated the amounts of diamide and DTT that are required for full oxidation and reduction of the Grx1-roGFP2 sensor *in vivo*, respectively. Figure S1 is now mentioned in the text at the position where we previously referred to Figure 1H for the first time. Thus, the order of the panels in Figure 1 does now correspond to the order in which they are mentioned in the text.

We added the time points to the individual panels in Figure 1G.

#2/ *As Figures S2 and S3 are not mentioned in the manuscript, they can be deleted.*

We deleted figures S2 and S3.

#3/ *Figure 7 needs a short explanatory legend.*

We now added an explanatory legend to the model previously presented in figure 7 (now Figure 9). It reads:

“Figure 9. Model for the dynamics, interplay and physiological impact of the cytosolic and mitochondrial glutathione pools. (A) Fast crosstalk via porins in the OMM takes place between the glutathione pools of the IMS and the cytosol. Because of this dynamic glutathione exchange the cytosolic glutathione reductase system exerts the major influence on the composition of the IMS glutathione pool. Although the matrix relies on glutathione delivery from the cytosol for replenishing its glutathione pool, EGSH[matrix] is maintained by matrix-localized reducing systems as GSSG cannot be exported from the matrix. (B) The oxidoreductase of the IMS Mia40 is *in vivo* partially reduced. Its redox state is maintained by reducing influences from the local glutathione pool (and newly imported reduced substrates), and oxidizing influences from the sulfhydryloxidase Erv1. *In vivo* Erv1 can shuttle its electrons either directly to oxygen or via the respiratory chain. This latter pathway might only be required under conditions of low oxygen.”

#4/ *The spelling should use consistently English or American spelling (according to Journal policy), at present we have both, "analyzed" and "analysed".*

We corrected these inconsistencies.

#5/ *p. 3, line 19: delete "redox"*

The word “redox” has been deleted.

#6/ *In my opinion, the statement at the bottom of p. 6 should be qualified (e. g., "did not significantly affect...") as the 30 min treatment with diamide potentially shows a very weak growth inhibition with the two highest dilutions (Figure S1).*

As suggested by the referee we corrected the statement in the text that refers to former figure S1 (now Figure S2). It now reads: “did not significantly affect”.

#7/ *Legend to Figure 3A: Define Zwf1.*

#8/ *Legend to Figure 2: The abbreviations Pgk1, Act1, MrpL36 and Mia40 need to be defined.*

#9/ *Figure S4: SOD and CCP1 need to be defined.*

#10/ *Figure 6: Define Erv1*

#11/ *p. 11, line 8 from bottom: italicize "N" in N-ethylmaleimide*

These abbreviations (#7 – #10) are the respective yeast standard names and follow the approved gene nomenclature. We now additionally defined them in either the text or the appropriate figure legends. The “N” in N-ethylmaleimide has been italiced.

Referee #2

#1/ *The influence of the respiratory chain and reactive oxygen species on the glutathione redox buffer remains of high importance. The authors only touched this issue in the course of their analysis in the cells treated with the inhibitors antimycin A, KCN or paraquat. However, these measurements seem to be done after growth under fermentative conditions characterized by little or no respiration. To really conclude about the effects of the inhibitors, the authors should perform the measurements under respiratory conditions. Also, it would be interesting to compare the glutathione redox potentials of the cells lacking mitochondrial DNA (no respiration, no respiratory chain complexes). These additions would lead to better understanding the role of the respiratory complexes in redox homeostasis.*

The referee raised a very important point, and we performed a series of experiments to address the link between the activity of the respiratory chain and EGSH. First, we compared EGSH at steady state and its recovery after oxidative shock in cells grown on the fermentable carbon source galactose and on the non-fermentable carbon source glycerol (novel Figure 5).

We thereby find that the OxD[matrix] becomes slightly more oxidizing in cells grown on glycerol, while the other compartments are unaffected.

Next, we extended the studies on inhibitors of the respiratory chain by testing them on cells grown on glycerol (novel Figures 5 and S4). Moreover, we also performed experiments using deletion mutants that affect the different complexes of the respiratory chain (novel Figures 5 and S6). The latter measurements had to be performed using galactose as a carbon source. We thereby find that cells grown on glycerol and treated for a short time with antimycin A exhibit a significantly elongated lag phase of the oxidant recovery curve in all three compartments! This is in contrast to the same treatment of cells grown on galactose where the lag phase only becomes elongated in the matrix. However, our experiments also show that the IMS – if at all – is affected to the same extent as the cytosol indicating that the cytosol and its reducing machineries exert the major influence on EGSH[IMS].

#2/ *The authors demonstrate that glutathione, in addition to the sulfhydryl oxidase Erv1, contributes to the redox state of MIA40 required for the transport and assembly of intermembrane space proteins. The role of Erv1 in maintaining the redox state of Mia40 is well known, and indeed confirmed by the authors in the current manuscript in the intact cells. What is the glutathione redox potential in the strains with depletion or overexpression of Erv1? Furthermore, the redox state of*

Mia40 has been demonstrated earlier to be dependent on the mutations in the respiratory chain complexes due to the fact that Erv1 passes the electrons derived from the Mia40 oxidation into cytochrome c and further to complex IV and molecular oxygen. So, in general complex III mutants have been shown to have more oxidized Mia40, whereas mutants in cytochrome oxidase (IV) rather opposite. It would be interesting to recapitulate these effects in vivo and also to measure the glutathione redox potential for mutant representatives, which were previously reported to affect redox state of Mia40.

As suggested by the referee we tested the influence of components of the pathway for disulfide bond formation in the IMS on EGSH. To this end, we upregulated Erv1 and Mia40 respectively by means of a regulatable promoter (novel Figures 5 and S8). We thereby find that the overexpression of Erv1 resulted in an increase of OxD[IMS] at steady state. Moreover, the recovery curve also recovers to this more oxidized OxD in the IMS. Notably, this increase in sensor oxidation is not due to a direct oxidation of the sensor by Erv1 as tested using purified components (novel Figure S8). Upregulation of Erv1 is the only case in which the IMS probe exhibits a different behavior compared to the cytosolic probe.

As correctly pointed out by this referee previous data demonstrating the coupling of Mia40 and Erv1 to the respiratory chain had only been obtained by *in vitro* studies (Bihlmaier et al [J Cell Biol 2007]; Allen et al [J Mol Biol 2005], Farrell et al [Biochemistry 2005], Dabir et al [EMBO J 2007]). We thus employed our *in vivo* approaches to recapitulate these findings *in vivo*. We thereby obtained a series of very interesting insights: (1) at ambient oxygen concentrations (20%) *RIP1* and *COX17* deletion mutants that result in inactive complexes III and IV exhibit the same Mia40 redox state as the wild type (novel Figure 7E). (2) At 1% oxygen concentration the Mia40 in *COX17* deletion cells is more reduced compared to wild type and *RIP1* deletion cells (novel Figure 7E). Thus, the respiratory chain is only required for Mia40 re-oxidation under oxygen-limited conditions. (3) In a strain depleted of Erv1 (GalL-Erv1 cells grown on glucose) the Mia40 redox state becomes predominantly reduced (Figure 7D). If we delete *GLR1* in this strain the Mia40 redox state remains unchanged (novel Figure 8C). However, when analyzing the recovery of the GalL-Erv1/D*glr1* strain after oxidative shock it is clearly impaired compared to a strain only depleted of Erv1 (novel Figure 8E). Thus, we provide clear evidence for a role of cytosolic glutathione reductase in the maintenance of a partially reduced Mia40 redox state in the IMS. We have thus defined for the first time the oxidizing and reducing influences that act on Mia40 in living cells.

#3/ *It would be very helpful to construct a table, in which the various measurements of the redox potentials for the cellular compartments are presented, including the data obtained in this study.*

As suggested by the referee we now constructed such a table. This table can be found in the supplementary information as Table S1.

#4/ *What is SS on the Figure 6B and 6D? Figure 6D: there is no sample without Dia, however the legend says otherwise.*

The labeling “SS” always refers to the “steady state” of the sensor and of Mia40. This has now been indicated in all figure legends. Moreover, we corrected the legend for Figure 6D so that it does not refer to a sample without diamide.

Referee #3

#1/ *Surprisingly the large TOM pore cannot compensate for the porin deletion in GSH equilibration. The authors did not discuss why TOM does not appreciably compensate for the lack of porin in GSH trafficking as this has been documented earlier for other small molecules.*

Indeed the TOM pore does compensate for the lack of Por1 as has been shown in many previous studies (*e.g.* Kmita et al, J Bioenerg Biomembr. (2004)). However, many of these – mainly *in vitro*/on isolated mitochondria performed – studies demonstrated that the kinetics of transport are significantly delayed compared to the wild type (*e.g.* Lee et al, J Bioenerg Biomembr. (1998)). We

think that this explains the differences found at steady state in our *in vivo* studies between D*por1* and wild type cells.

#2/ *The authors should mention the pioneering work of Hackenbrock from the 70s and early 90s on the ionic strength similarities of the intermembrane space and the cytosol.*

We now cite the work from Hakenbrock in the context of the free permeability of the OMM (p. 3)

2nd Editorial Decision 16 May 2012

Thank you for the submission of your revised manuscript. As you have properly addressed the concerns originally raised by the referees in the first round of review, I am writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few more minor details have been addressed, as follows.

Browsing through the manuscript, I have noticed that the statistical representation of the data in figures 7D and E is not properly described. The number of independent assays performed as well as the significance of the bars depicted in the figure must be stated in the figure legend, as you have correctly done for the other figures. Along these lines, I have the impression that the legend to figure 8C actually corresponds to panel 8D and vice versa. Could you please confirm this so we can correct it if necessary? Similarly, just reply to this email with the statistical description for panels 7D and E and we will add it to the figure legends.

As a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures?

The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

After these remaining corrections have been introduced, you will then receive an official decision letter accepting your manuscript for publication in The EMBO Journal. This letter will also include details of the further steps you need to take for the publication process to continue.

Thank you for your contribution to The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #2

The authors adequately fulfilled the referee's points. Altogether, this is a comprehensive and important work that addresses glutathione redox potential in various cellular compartments and its dynamic changes upon modifications of the redox processes. Certainly, this work will form a solid basis for further deciphering the redox networks operating in the cell and will help to properly interpret various effects related to cellular redox homeostasis.