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Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control

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1st Editorial Decision 05 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, we experienced difficulties in finding suitable and willing referees for this manuscript; and one of the referees was not able to get back to us with his/her report as quickly as initially expected. Your manuscript has now finally been seen by three referees whose comments are shown below. As you will see while all three referees consider the study as highly interesting in principle, it also becomes clear that stronger evidence for your key conclusion - that GSH is not essential as a redox buffer, but rather because of its requirement for cytosolic ISC assembly - would be required. Given that referees 1 and 2 are more positive overall, I have come to the conclusion to give you the chance to address the referees' concerns by revision. However, it will be important to address the referees' concerns in an adequate manner and to their full satisfaction. I should remind you that it is 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 as well as on the final assessment by the referees. A final decision can only be made at that stage.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

I have read and reviewed the manuscript entitled "Glutathione revisited: a vital function in iron metabolism but an ancillary role in thiol-redox control", submitted by Michel Toledano and colleagues for publication in the EMBO Journal.

Most of the individual findings of this work have already been proposed or published. The authors complement prior data with few experiments to provide a revision, more than an original article, with very interesting conclusions and outcomes. My particular opinion is that their'integration' job may be worth publication in EMBO Journal, after some editorial changes and new experimental contributions to support his main hypothesis: that glutathione is essential not as a redox buffer but rather due to its role in cytosolic ISC assembly.

Major points

1. One of the biggest issues of this manuscript is that cells with an overload of GSH have defects in ISC assembly, as cells depleted in GSH do. Since most of the paper relies on the first experimental model (o/e HGT1 cells), authors should show some experimental data to justify it. For instance, if sequestration of glutaredoxins by an excess of GSH may be the problem (as stated in the discussion), can they ameliorate damage or decrease FET3 expression by o/e of Grx3 in HGT1 cells?

2. Page 12, discussion: "The molecular function of GSH in the export pathway does not seem to involve its thiol-reducing activity". What is that observation based on? What do they mean?

3. Characterization of delta gsh1 cells (figs. 5-7). How to explain that not only GSH, but also DTT, rescues the growth of delta gsh cells (Sipos et al 2002 and Fig. 5B)? Why do they need 1 mM GSH to support the growth of delta gsh1 cells, if only a small concentration would be required to compensate the defects of ISC assembly? According to their model, one should expect that the GSH concentration to allow growth of a delta trr1 strain (Fig. 6A; see below) should be higher than that of a delta gsh1 strain (Fig. 5B). Combination of extracellular iron and GSH should be synergistic on growth (Fig. 5A)? To confirm the phenotype of delta gsh1 cells, they should show aconitase (preserved) and leu1 (decrease) activities, as shown for HGT1 cells. We believe that the decrease in leu1 activity in delta gsh1 cells is show in Supplementary Fig. S6, but this figure is not even mentioned in the results sections. It should be moved to the manuscript. As you will see below, THE FIGURES ARE A REAL MESS WITHIN THIS MANUSCRIPT.

4. To characterize the role of GSH as a backup for thioredoxin in thiol maintenance, they should show Fig. 6A also under anaerobic conditions, with the prediction that delta trr1 cells should grow better and require less extracellular GSH to sustain growth, and the same amounts (or similar) of GSH should suffice growth of delta gsh1 cells. Also, is delta trr1 growing better with o/e of HGT1?

5. The relationship between h2o2 signalling, thioredoxin oxidation, GSH depletion and induction of iron starvation (i.e. induction of FET3) (Fig. 7C) is very poorly established. We propose to eliminate such figure, or to alternatively show the same kinetics after h2o2 imposition for: h2o2 scavenging, trx oxidation, GSH depletion and Leu1 inactivation.

6. Fig. 5B and Fig 3D. How can supplemental iron have such a different effect on the two model systems used in this manuscript? Why can Fe alleviate the ISC assembly defects in delta gsh1 cells, but not in the HGT1 model?

7. Since one of the major outcomes of this manuscript is to define that the major role of GSH is in cytosolic ISC assembly and not in redox maintenance, they should further complement this hypothesis with genetic experiments to confirm that conclussion: (i) is delta gsh1 synthetic lethat with mutations in genes coding for mitochondrial or cytosolic ISC?; (ii) o/e of ISC machinery components can suppress the defects of delta gsh1 cells?.

8. Regarding microarray experiments, the major discrepancy between the actual data and what is written in the results section is the induction (or not) of Yap1 target genes in delta gsh1 cells (suppl Table S3). Induction of those genes seems to be quite strong in the microarray data (block 2 of Table S3 - mistakenly annotated as Table S4): 10-fold, 19-fold, 7-fold... for up to 10 genes. It is important, because the authors claim that redox is not greatly altered in this strain, and that is why there is a 'very minor activation of Yap1, if any". We disagree. They should eliminate such sentence, and answer: (i) why Yap1-dependent gene expression is not triggered in HGT1 cells, as it is in delta gsh1; (ii) they also say that there is a "lack of perceptible Yap1 oxidative activation"; show it.

Minor points

1. Fig. 6. Are cells lacking thioredoxins (and not trr1) also having high GSH levels? And enhanced FET3 expression? They should also measure concentration of GSH in delta trr1, even though it has been published.

2. Page 7, results: where does PF1 come from? They provide a manuscript, not a vendor, but the main author is not in the acknowledgments.

3. Fig. 4F: what is the cause of h2o2 production by the ER?

4. Can they reconcile their hypothesis with the fact that frataxin-deficient cells (deficient in mitochondrial ISC assembly) have 5-times the amount of GSH than WT cells? (Human Mol. Genet. 2008, 17:2790).

5. Figures are improperly labelled: where is Fig 6, E (page 9)? Also in page 9, Fig. 6D is Fig 7A, and Fig 6E of page 10 may be Fig. 7B, and 6F is 7C. A mess. In figure 7B, hr should be min, I guess. There are many mistakes in the text while referring to figures and tables. Here are some: page 5 it says figure S3 instead of supplementary table S3; page 8: it should say Table S3 (but the Table is called S4, change it too).

6. Abstract, second line: 'To determine which of these functions...'. They have not established earlier in the text that there are several functions, only its role in ISC assembly. Re-write.

7. We are concerned about the growth and survival curves to GSH in HGT1 cells (Fig. 1B,C). What happens with the cells that do survive, are they the ones resuming growth after GSH depletion? Why survival stays at 40% even at higher doses of added GSH? It would be nice to know the intracellular concentration of GSH at 200 μM (Table 1), since the growth and survival of cells at this concentration is shown in Figure 1B and C. The authors should discuss why increasing the concentration from 50 μM to 200 μM GSH does not seem to result in decreased survival (Fig 1C) or why cells that do reach a similar OD after 25 h of treatment with increasing concentration of GSH (albeit with a small delay at 200 μM) then do not totally survive. It seems as GSH would have short term and long term effects.

8. Other differences between the transcriptome profiles of delta gsh1 and HGT1 cells (besides the already mentioned ER response and Yap1 dependent genes) is that the former does not (i) display major repression in ribosome biogenesis/translation (even though cells are dying), (ii) show altered carbohydrate metabolism. Explain.

9. Again regarding microarray data, in Figure 2 Legend, or either in the text, it should be described in more detail how the genes were assigned to functional categories, for instance which database was used.

10. More editing: Main text, page 6, lines 12 to 15: indicate which figure represents the stated affirmation; Figure 3D: indicate the GSH concentration at which the experiment was performed (in all cases, substitute the sentence 'at the indicated concentrations' by the actual concentration, is shorter and more useful); Figure legend 4F: not correctly explained; Figure 5B: indicate the concentrations of GSH, DTT and FeCl3 at which the experiments were performed; Figure 7/figure legend 7: indicate the h2o2 concentration at which the experiment was performed (it is only stated in results); supplementary table legends of Table S1 and Table S2, lines 1 and 2 in both cases, change the concentration of GSH from 50 mM to 50 μM; Figure legends of Supplementary figures S3 and S4, line 2 in both cases change the concentration of GSH from 50 mM to 50 μM; Supplementary figure S7: include the line that represents the data of the strain ire1Δ (HGT1); in Figure S1 A, the numbering of the Y axis should follow the same format.

11. In Table I, second column, the concentration of GSH at 100 μM should follow the same format as for the other lines, i.e. GSH (100 μm).

12. Figure 6A does not seem to correspond to spot dilutions as described in the figure legends, but rather to plating a determined amount of cells onto plates with different GSH concentrations.

Referee #2 (Remarks to the Author):

In this provocative study, Kumar et al address an old question, the role of glutathione in yeast cells. Most textbooks state that GSH -a small tripeptide present at high concentrations (up to 10 mM) in all eukaryotic cells- is one of the major redox buffers. Indeed, loss of GSH has been often associated with increased susceptibility to oxidative stress. GSH is required also for the synthesis of iron-sulfur clusters (ISC). To determine which of the two functions (redox buffering or ISC maturation) underlies the requirement of GSH for yeast viability, the authors design an interesting strategy to cause either depletion or overproduction of the tripeptide. The results demonstrate that in both conditions an intense response is elicited that mimics iron-starvation conditions. The authors conclude that the primary function of GSH is to assist ISC maturation, high concentrations being needed to insulate this essential step from redox oscillations. The thiol-redox buffering role is viewed as an ancillary, back-up function of GSH.

Overall, the results presented in the paper are convincing and intriguing. There are however a number of points that the authors should take in consideration before the Ms can be considered for publication in the EMBO J.

Main criticism

- To unequivocally demonstrate that GSH toxic levels inhibit exclusively cytoplasmic Fe-S cluster maturation, w/o affecting mitochondrial physiology, yeast cells should be grown in a medium containing a respiratory carbon source (glycerol) rather then a fermentative carbon source (glucose).

- The authors demonstrate an increase in ROS production in cells grown in the presence of high GSH levels. Since Fe/S cluster is destabilized by ROS, the author should clarify the contribution of reactive oxygen species in the impairment in cytosolic Fe/S cluster. Is FET3 mRNA upregulated when cells are grown at high GSH levels, in the presence of a ROS scavenger?

- In the second part of the manuscript, the authors describe the alteration of iron metabolism in conditions of GSH depletion. To better investigate how mitochondrial Fe/S cluster synthesis is impaired by the absence of GSH, yeast cells should also be grown in the presence of a respiratory carbon source.

- Recently, a paper describing the critical role of GSH in maintenance of mtDNA and iron homeostasis has been published (Ayer et al., Free Radical Biology and Medicine, doi: 10.1016/j.freeradbiomed.2010.09.023). The results presented by the authors must be re-evaluated, and more emphasis should be given to the novel implications with respect to this publication.

- The dual role of GSH in ISC maturation and redox buffering poses an interesting evolutionary problem. The authors might wish to embark in a few speculations concerning what came first.

Minor points

- In many sections, the writing can be improved considerably. As presented, it is not linear enough to attract a wider readership.

Referee #3 (Remarks to the Author):

The manuscript by Kumar et al. entitled "Glutathione revisited: a vital function in iron metabolism but an ancillary role in thiol-redox control" describes interesting observations in yeast cells exposed to toxic or sub-optimum levels of glutathione. The authors show that both conditions result in stimulation of Aft1/2 regulated gene expression, suggesting that each condition elicits an iron starved response. The effects of sub-optimum glutathione have been documented previously, but the effects of toxic glutathione levels on iron related gene expression and metabolism represent novel findings. The manuscript also addresses whether the essential function of glutathione is related to Fe-S cluster biogenesis or its redox function and concludes that the essential function of glutathione is its role in Fe-S cluster biogenesis. Overall, the results in this manuscript would be of interest to a general reader were they to provide mechanistic insight into the role of GSH in iron metabolism and/or its redox function.

Through genome-wide analysis, the authors show that toxic levels of GSH achieved through over-expression of HGT1 and addition of excess glutathione alters expression of genes related to iron metabolism and to ER stress and the unfolded protein response. The latter set being strongly induced as well as the subset of iron related genes that are controlled by the Aft1 and 2 transcription factors. While Aft1/2 controlled and ER stress/UPR genes are both induced under GSH excess conditions, this effect appears to be through responses to different signals as the kinetics of induction differs for these two classes of genes. The effect on Aft1/2 controlled genes suggests that a pseudo-iron starved condition was established in cells with toxic levels of GSH. It seems likely that this effect of excess GSH is the result of damage to Fe-S clusters in the cytoplasm. It has been reported by other investigators that Aft1/2 sense cellular iron status through a cytosolic Fe-S protein. The effect of excess GSH may well be through damage to such an Fe-S protein. While the authors provide correlative results on GSH accumulation and activity of mitochondrial and cytoplasmic Fe-S proteins, experiments that provide more mechanistic information are needed to increase insight into this important question.

Others have reported that yeast deficient for GSH show defects in extramitochondrial Fe-S cluster biogenesis and respond to such deficiency by upregulating Aft1/2 controlled gene expression. In this manuscript the authors also seek to address the cellular requirement for GSH and to link this requirement to thiol-redox control or cellular iron metabolism, specifically Fe-S cluster metabolism. Transcript profiles in GSH deficient cells are compared to normal cells and to cells deficient for iron or the mitochondrial ISC export function. This last condition impairs cytosolic Fe-S cluster biogenesis. The authors find great similarities in the transcript profiles from cells in these three conditions and argue that since alteration in iron metabolism gene expression is the only defect linked to GSH depletion that GSH essentiality must be linked to this role and not a thiol-redox function. Gene array data do not address individual gene function or absolute level of expression. However, analysis of the Aft1 controlled gene FET3 showed its expression to be greatly elevated in gsh1 yeast, similar to the reports of others. Addition of iron rapidly reduced the high FET3 expression of GSH deficient yeast and partially rescued growth of gsh1-delta cells in media lacking GSH. The authors should be cautioned that FET3 mRNA levels may not reflect only Aft1 activity but is also a reflection of mRNA degradation, which for FET3 mRNA is rapid in the presence of

excess iron. The use of a reporter gene linked to the FET3 promoter would help the authors to sort out such questions. The ability of DTT to rescue growth of gsh1 yeast further complicates interpretation of these results. Prior investigations have concluded that this ability of DTT to rescue gsh1 yeast demonstrates a thiol-redox function of GSH. Again, without mechanistic insight, it is not possible to distinguish between these two possibilities, although the authors do clearly show that much less GSH is required for cell viability than for general redox control.

Kumar et al. further investigate the role of GSH in thiol-redox maintenance, and examine whether it functions in this role as effectively as thioredoxin. The results presented in the manuscript show that GSH is not as effective in controlling thiol-redox as thioredoxin, leading the authors to propose that it serves only a backup function to thioredoxin in redox control. They further enquire whether GSH provides a link of thiol-redox to iron metabolism. They present interesting results that show a modest increase in FET3 expression in a trr1-delta strain. This is consistent with their notion that GSH is limiting in these strains even though absolute levels of glutathione are higher by 2-3 fold. Addition of GSH to trr1 yeast suppressed FET3 expression and restored growth to near normal level at high GSH concentration. Much lower levels of GSH were required to rescue growth of gsh1 yeast, leading the authors to the conclusion that GSH is essential because of a non-redox control function(s).

Additional points:

1. The addition of iron to yeast with toxic levels of glutathione did not protect. Does the reduction of iron have a protective effect or is it detrimental?

2. In figure 3D was 100 and 250 millimolar FeCl3 really used for this experiment?

3. Page 10, line 7: (fig. 6F) should read (fig. 7C).

4. It would be helpful to add wild type plus GSH to Table I.

1st Revision - authors' response 21 December 2010

RESPONSE TO REFEREES

The manuscript has been edited according to the referee's requests.

In the figures, we introduced the following changes:
- Fig. 5: new figures B, D and E; fig. C moved to supplementary fig. S7B.
- Fig. 6: a new fig. B.
- Fig. 7, C removed

In the supplementary figures we introduced the following changes:
-Fig. S4: two new figures C and D)
-Fig. S5: 3 new figs D, E and F and a new panel in C;
-Fig. S7: former fig. 5C as fig. B.
-Fig. S8: removed, as unnecessary

We also provide an accompanying document for referee-only, containing extra-figures A to G, and response to referee 1, minor point 1. (Data not included in the Peer Review Process File.)

1. REFEREE 1. We thank this referee for his/her thoughtful comments on the manuscript, to which we provide the following answers:

MAJOR POINTS

1. ***Since most of the paper relies on the first experimental model (o/e HGT1 cells), authors should show some experimental data to justify it. For instance, if sequestration of glutaredoxins by an excess of GSH may be the problem (as stated in the discussion), can they ameliorate damage or decrease FET3 expression by o/e of Grx3 in HGT1 cells?***

Action:

As requested, we have now monitored the effect of overexpressing Grx4 in HGT1 on both growth and FET3 expression. Data are included as supplementary fig. S5, D, E and F. Grx4 with a His tag at the C-terminus expressed from a 2-μ plasmids allowed about a fourfold increase in protein abundance compared to when expressed from a centromeric plasmid (fig. S5, D). Further both tagged and untagged versions of Grx4 expressed from both 2-m and centromeric plasmids all similarly rescued the growth defect of grx3Δgrx4Δ. We used His-tagged Grx4 expressed form a 2-μ plasmid, which corrected the high FET3 expression triggered by GSH, but only at low non-toxic concentrations of GSH (5 and 10 μM) (E). However, overexpressed Grx4 also slightly increased GSH toxicity (F). These data cannot be used to definitely prove or rule out that GSH titrates glutaredoxins; however they necessarily indicate that GSH toxicity result from other mechanisms, such as a general and direct effect on Fe-S enzymes, or the exacerbation of a yet unknown function of GSH in iron metabolism, as initially discussed. The new fig. S5 is now cited in discussion, paragraph 2.

2. ***Page 12, discussion: "The molecular function of GSH in the export pathway does not seem to involve its thiol-reducing activity". What is that observation based on? What do they mean?***

Answer:

The role of GSH in iron metabolism does not seem to involve its thiol-reducing function: that is GSH reducing a disulfide —either indirectly via dithiol glutaredoxin (Grx1/Grx2) or, less likely, directly— or forming an adduct with a Cys sulfhydryl (*S*-thiolation). Our assertion is based on a set of observations:

(i) The GRX1 and GRX2 double mutant (grx1Δgrx2Δ) does not constitutively express *FET3*. As measured by QT-RT-PCR, FET3 expression in this mutant was actually lowered by one third with regard to wild type, and not increased. If indeed the function of GSH in iron metabolism was to reduce a disulfide through dithiol glutaredoxins, the loss of these enzymes should have phenocopied GSH depletion, with regard to *FET3* expression.

(ii) Excess GSH favors reduction of disulfides—this is indeed the effect of excess GSH in the ER (fig. 4). Accordingly, if the thiol-reducing property of GSH were to be involved in iron metabolism, we would not expect excess GSH to alter it, as we observed.

(iii) Reciprocally excess GSSG favors disulfide bond formation. If the thiol-reducing property of GSH were to be involved, GSSG should alter iron metabolism. We directly tested whether GSH or GSSG alters iron metabolism. We exposed HGT1 cells to GSSG (50 μM) and measured total GSH and GSSG levels, and FET3 expression (fig. Referee A, for referee-only. Data not included in the Peer Review Process File.) After 15 min, GSSG increased up to 25 mM, while GSH remained low at 7 mM; after 30 min, both GSSG and GSH levels increased to 60 mM and 25 mM, respectively, suggesting that the GSH levels increase resulted from GSSG reduction. *FET3* expression was not induced from the 15 min but from the 30 min time point (fig. Referee B, data not included in the Peer Review Process File). In contrast, 15 min after incubation with GSH (100 μM), GSH levels were > 60 mM and was FET3 was induced by > 2 fold. These data indicate that it is reduced and not oxidized GSH that induces FET3.

(iv) Only 0.5 μM GSH are required for wild type growth of gsh1Δ (fig. 6)(about 1% of the wild-type amount of GSH, see table 1), and 2 μM during 30 min for halting Aft1 activation in this strain (not shown), which favor the idea that GSH is not acting in thiol reduction: indeed according to the Nernst equation, at this concentration, the GSH redox potential is very oxidizing (~ 150 mV) (see also (Ayer et al) and GSH should behave as a thiol-oxidant and not as a reductant.

(v) DTT did not correct the defective cytosolic Fe-S cluster maturation of gsh1Δ cells (Sipos et al, 2002), which should be expected if GSH had a thiol-redox function in this pathway.

(vi) A thiol-reducing function of GSH is unlikely when considering that one likely function of GSH in iron metabolism involves a partnership with monothiol Grxs (Grx3, Grx4) with which it forms instable Fe-S clusters, as discussed in the paper. Accordingly, GSH would need to be in the reduced form, not for reducing a disulfide, but for serving as ligand of a Fe-S cluster.

(vii) Lastly, and importantly, the rescue by iron of the GSH auxotrophy of gsh1Δ is by itself a very strong indication that the essential requirement of GSH for viability is linked to its function in iron metabolism and that such function does not depend on the thiol-reducing function of GSH (see also response to major point 3). We have now repeated these iron rescue experiments and provide new results strengthening our initial claim (see also referee 2, fourth query).

Action:

We have added sentences summarizing the points (iv), (v) and (vii) in the discussion, second paragraph.

3. ***Characterization of delta gsh1 cells (figs. 5-7). How to explain that not only GSH, but also DTT, rescues the growth of delta gsh cells (Sipos et al 2002 and Fig. 5B)? Why do they need 1 mM GSH to support the growth of delta gsh1 cells, if only a small concentration would be required to compensate the defects of ISC assembly?***

Answer:

(i) The referee was mislead by figure 5 that indeed used GSH at 1 mM to rescue the growth of GSH-depleted cells, probably because the amounts of supplements were only mentioned in the legend of fig. 5, A. However, figure 6 then shows that GSH at concentrations below the micromolar (0.5 μM) can fully rescue the growth of these cells, in keeping with the proposed model.

(ii) With regard to the DTT rescue of GSH-depleted cells, the amount of DTT needed (300 μM) (Grant et al, 1996) (our unpublished data) is 600 fold higher than that of GSH (0.5 μM) (this work). Further, rescue is very poor compared with the one observed with iron, and is clearly observed only with cells still carrying some remaining GSH, in contrast to the iron rescue of these cells (see new fig. 5D, E). We explained in the paper (see page 13, fourth paragraph of the discussion) that DTT presumably substitutes for some of GSH redox functions (our unpublished data suggest that these redox functions are mostly linked to the ER, but this is beyond the scope of this paper), thereby decreasing its consumption rate, as we previously suggested (Sharma et al, 2000; Spector et al, 2001), which has a sparing effect on the remnants of GSH molecules that remain available for the vital iron function. In other words, when GSH levels are very low DTT decreases the crosstalk existing through GSH between iron and redox metabolisms, as presented in fig. 7. A paper published while this was under review showed that DTT only provided 2 and half divisions of GSH-depleted cells, and also concluded DTT has a sparing effect on GSH (Ayer et al).

(iii) In *E. coli*, a strain lacking thioredoxin reductase and glutathione reductase (*trxB gor*) is unviable due to defective reduction of RNR; however growth is full rescued by DTT (Gon et al, 2006; Ortenberg et al, 2004), which indicates that if the inviability of GSH-depleted cells was linked to defective thiol-reduction (as in bacteria), it should be fully rescued by DTT, which is not the case.

Action:

We mentioned the amounts of supplements in the legend of all figures. We also included point (iii) in the discussion section.

According to their model, one should expect that the GSH concentration to allow growth of a delta trr1 strain (Fig. 6A; see below) should be higher than that of a delta gsh1 strain (Fig. 5B).

Answer:

Yes, this is exactly what is shown in figure 6A: compared to gsh1Δ (0.5 μM), 100 fold more GSH is needed for growth of trr1Δgsh1Δ cells to near wild-type levels. The use of the trr1Δ gsh1Δ double mutant is the only way how to rigorously comparatively quantify the GSH need of trr1Δ and wild type strains.

Combination of extracellular iron and GSH should be synergistic on growth (Fig. 5A)?

Action

As mentioned in point 2, we repeated the iron growth rescue experiments and provide new results strengthening our initial claim (see also referee 2, fourth query). Accordingly, we found that the iron rescue was much more potent with gsh1Δ cells made petite (non-respiring), a change that also cleared the difference in growth rescue between aerobiosis and anaerobiosis. We thus here used non-petite gsh1Δ cells under aerobiosis to favor the observation of an iron/GSH synergistic effect. GSH at the suboptimal concentration of 0.05 μM promoted a very weak growth of gsh1Δ, as did FeCl3 at 100 μM under aerobiosis. However the combination of both GSH and FeCl3 was more efficient (fig. Referee C). We cannot conclude however, whether the effects of the two supplements are additive or synergistic.

To confirm the phenotype of delta gsh1 cells, they should show aconitase (preserved) and leu1 (decrease) activities, as shown for HGT1 cells. We believe that the decrease in leu1 activity in delta gsh1 cells is show in Supplementary Fig. S6, but this figure is not even mentioned in the results sections. It should be moved to the manuscript. As you will see below, THE FIGURES ARE A REAL MESS WITHIN THIS MANUSCRIPT.

Answer:

We apologize for the trouble caused by mislabeled figures and by omitting mentioning supplementary fig. S6 in the text. This figure was not included as showing published data (Sipos et al, 2002).

Action:

As requested, we have now moved supplementary fig. S6B into the manuscript as fig. 5A, and mentioned it in the results, paragraph 5.

4. ***To characterize the role of GSH as a backup for thioredoxin in thiol maintenance, they should show Fig. 6A also under anaerobic conditions, with the prediction that delta trr1 cells should grow better and require less extracellular GSH to sustain growth, and the same amounts (or similar) of GSH should suffice growth of delta gsh1 cells.***

Action:

We monitored the growth of WT, trr1Δ, gsh1Δ, and gsh1Δtrr1Δ under anaerobic conditions (new fig. 6B).

(i) As the referee predicted, trr1Δ grew to near wild type levels without added GSH, and adding GSH did not improve growth further.

(ii) The amount of GSH needed for wild type growth of gsh1Δ was strictly similar under anaerobiosis and aerobiosis (0. 5 μM) (see fig. 6A and B).

(iii) Under anaerobiosis, 10 μM GSH promoted growth of gsh1Δtrr1Δ to the level of the trr1 strain, which is 20 fold less GSH than what was needed under aerobiosis (200 μM) (see fig. 6A and B). Importantly, this strain still required more GSH than the single gsh1Δ mutant (10 vs 0.5 μM). These data indicate that a large part of the redox load applied on GSH in trr1Δ is oxygen-dependent, but that a smaller part exists that is not O_2-dependent. Therefore, the fact that under anaerobiosis, trr1Δ does not need extra-GSH (new fig. 6B) indicates that the compensatory increased GSH levels of this strain suffice to sustain the O_2-independent redox load of the strain.

In addition to new fig. 6B, the result (paragraph 6) and discussion (paragraph 3) sections were edited.

Also, is delta trr1 growing better with o/e of HGT1?

Answer:

HGT1 overexpression in trr1Δ was highly toxic before adding GSH to the medium. HGT1 being a plasma membrane protein using the secretory pathway, we thought that its overexpression might be toxic in trr1Δ that suffers from constitutive ER stress for reasons that have not been yet clarified (Trotter & Grant, 2002). However, overexpressing in trr1Δ several genes encoding non-secretory proteins was also highly toxic. The same effects were seen after several transformation attempts and cannot be rationalized at the present time.

Action:

The experiment could not be performed for the reason cited above.

5. ***The relationship between h2o2 signalling, thioredoxin oxidation, GSH depletion and induction of iron starvation (i.e. induction of FET3) (Fig. 7C) is very poorly established. We propose to eliminate such figure, or to alternatively show the same kinetics after h2o2 imposition for: h2o2 scavenging, trx oxidation, GSH depletion and Leu1 inactivation.***

Action:

We removed this figure, as suggested by the referee, as it was indeed not fully documented, and also since it now overlapped with the new data under anaerobiosis.

6. ***Fig. 5B and Fig 3D. How can supplemental iron have such a different effect on the two model systems used in this manuscript? Why can Fe alleviate the ISC assembly defects in delta gsh1 cells, but not in the HGT1 model?***

Answer:

This question needs to consider that GSH depletion and toxic levels are mediated by different processes: the former is the loss of function by lack of GSH and occurs after overnight growth, while the later is a toxic effect of GSH, and occurs abruptly. In addition, GSH toxicity not only invokes an alteration of iron metabolism, but also of secretion, which makes comparing the HGT1 and gsh1Δ cells even more difficult. Further, considering GSH toxicity, the impact on secretion is dominant over the one on iron metabolism, as indicates a partial remediation of GSH toxicity by the thiol oxidant diamide (Igbaria et al., follow up study in preparation; diamide decreases the reductive stress imposed on protein oxidative folding in a narrow range of concentrations), and the exacerbation of GSH toxicity in the ire1Δ strain (fig. 4H). Accordingly, one

may only see effects worsening (as excess iron) but not decreasing the GSH toxic impact on iron metabolism, as such effect would be offset by the remaining dominant impact of GSH on secretion. Here are possible answers to the referee's question:
(i) Iron increases endogenous GSH production (Auchere et al, 2008) and probably does it also in HGT1 cells, which should exacerbate overall GSH toxicity.
(ii) In GSH depleted cells, there is a 20-fold increase of iron in mitochondria (Sipos et al, 2002), which might create a state of iron deficiency outside this organelle by sequestration, as suggested for other ISC assembly mutants (Chen et al, 2002), possibly explaining why exogenous excess iron improves growth. [Please, see also the response to referee 2, point 4: in gsh1Δ, iron also increases O_2-dependent loss mtDNA (Ayer et al, 2010) by accumulating in mitochondria]. In HGT1 cells, we speculate the abrupt rise of GSH disrupts Fe-S clusters, which may itself contribute to toxicity and trigger the release of cytosolic free iron that may also contribute to toxicity by growth inhibition, as shown recently (Lin et al). Adding large excess iron (>100 μM and above) might exacerbate this toxicity.
Action:

According to answer **(ii)**, inactivating high affinity iron uptake aft1Δ, aft1Δaft2Δ) should have decreased GSH toxicity, at least slightly, but did not (Supplementary fig. S5, C). However, we strongly believe that if these mutations could have limited the GSH toxic impact on iron metabolism, it would not be seen due to remaining GSH impact on secretion. Nevertheless, we retested strains with an inactive iron high affinity uptake, this time using aft1Δ, and also fet3Δ (Fet3 is part of the iron high affinity uptake system), as these mutants do not require exogenous iron for growth in contrast to aft1Δ aft2Δ; again none of these mutations limited GSH toxicity. We also tested the effect of desferroxamine (a membrane permeable iron chelator)(see response to referee 3, additional point 1), but this compound did not limit GSH toxicity either (not shown). The new experiment with the aft1Δ and fet3Δ strains has been added to supplementary fig. S5C, and the result section (paragraph 3) and discussion (paragraph 4) edited in line with the answers provided here.

7. ***Since one of the major outcomes of this manuscript is to define that the major role of GSH is in cytosolic ISC assembly and not in redox maintenance, they should further complement this hypothesis with genetic experiments to confirm that conclusion: (i) is delta gsh1 synthetic lethal with mutations in genes coding for mitochondrial or cytosolic ISC? (ii) o/e of ISC machinery components can suppress the defects of delta gsh1 cells?***
Answer:
(i) In fact, it has already been shown that mutations in *ATM1* and *GSH1* are synthetic lethal (Sipos et al, 2002), which was taken by these authors as an indication that these genes operate in the same pathway.
(ii) We previously conducted a genetic screen for genes whose overexpression would suppress the GSH auxotrophy of the gsh1Δ strain but we failed (unpublished, Spector D, Ph.D. thesis, 2001). We also conducted a genetic selection for extragenic suppressors of the GSH auxotrophy of gsh1Δ cells but did not identify any true suppressors (Spector et al, 2001).

8. ***Regarding microarray experiments, the major discrepancy between the actual data and what is written in the results section is the induction (or not) of Yap1 target genes in delta gsh1 cells (suppl Table S3). Induction of those genes seems to be quite strong in the microarray data (block 2 of Table S3 - mistakenly annotated as Table S4): 10-fold, 19-fold, 7-fold... for up to 10 genes. It is important, because the authors claim that redox is not greatly altered in this strain, and that is why there is a 'very minor activation of Yap1, if any". We disagree. They should eliminate such sentence***
Answer:

With regard to our interpretation of the Yap1-dependence of gene expression in supplementary table S3, and our claim that Yap1 is not induced upon GSH depletion, we must provide complementary informations:
(i) One should consider the number of genes of a pathway that are upregulated, more than amplitude of gene expression (often poorly quantified on microarrays). The mRNA profiles in yap1Δ and yap1Δskn7Δ cells exposed to H_2O_2 identified 87 Yap1 and 144 Yap1-Skn7 targets genes, and among these, antioxidant genes as *TSA1*, *TSA2* were among those with the highest induction (Biteau B., Ph.D. thesis, unpublished data). Similar results have been published (Gasch et al, 2000). Further, 70% of this large Yap1 regulon was upregulated in a thioredoxin reductase (trr1Δ) mutant (Carmel-

Harel et al, 2001). In comparison, the mRNA profiles of GSH-depleted gsh1Δ cells showed 11 Yap1-dependent genes upregulated, which represent a minor portion of the Yap1 regulon. Furthermore among these, 6 genes are also aft1-Aft2 targets (Fet5, Fre1, Sdl1, Oye3, Met2, Ecm4), a regulon that we know is strongly upregulated here. These data would support the existence of at best a minor activation of the Yap1 regulon.
(ii) Tsa1, a *bona fide* Yap1-dependent gene is strongly down regulated in GSH-depleted cells, which goes against the idea of an activation of Yap1 in this setting.
(iii) The fact that Yap1 is not oxidized in the gsh1Δ strain goes strongly against an activation of Yap1 (see below).
Action:

As requested, we rephrased our statement in the results, paragraph 5 by saying " these results show at best a minor activation of Yap1... and removing "if any". We hope this change will satisfy the referee.

Answer (i) why Yap1-dependent gene expression is not triggered in HGT1 cells, as it is in delta gsh1
Answer:

Yap1 is activated by oxidation by peroxide and is specifically reduced by thioredoxin. Inactivation of thioredoxin leads to defective reduction of Yap1, and its accumulation in the oxidized active form. Therefore, Yap1 oxidation can conceivably be triggered by depletion of GSH (see above), but not by an increase in its concentration.
No action

(ii) they also say that there is a "lack of perceptible Yap1 oxidative activation"; show it.
Action:

This experiment was performed and is now shown as fig. 5B. It shows that indeed Yap1 does not oxidize upon drastic GSH depletion.

MINOR POINTS (REFEREE 1)

1. ***Fig. 6. Are cells lacking thioredoxins (and not trr1) also having high GSH levels? And enhanced FET3 expression?***
Answer:

Please see the accompanying document for referee-only. (Data not included in the Peer Review Process File.)

They should also measure concentration of GSH in delta trr1, even though it has been published.
Action:

We have now added in the table the measures of GSH and GSSG in trr1Δ, and recalculated values for HGT1 cells.

2. ***Page 7, results: where does PF1 come from? They provide a manuscript, not a vendor, but the main author is not in the acknowledgments.***
Answer:

We thank the referee for pointing out this neglect. We actually made an acknowledgment for the gift of PF1 in the paragraph 5 of the method section but not in the acknowledgements per se. This has now been corrected.

3. ***Fig. 4F: what is the cause of h2o2 production by the ER?***
Answer:

The FAD-dependent ER oxidoreductin Ero1 was shown to transfer electrons from thiol substrates to molecular oxygen, and the reduction of molecular oxygen by Ero1p yields stoichiometric hydrogen peroxide (Gross et al, 2006). In the HGT1 cells, the upsurge of GSH in the ER both maximally activates Ero1 by reduction of its inhibitory disulfide, and provides an unlimited substrate supply, which we think perpetuate Ero1 cycling and hence H_2O_2 production.
Action:

We added a few words in the result section (paragraph 4) that explain why is H_2O_2 produced by actively cycling Ero1.

4. ***Can they reconcile their hypothesis with the fact that frataxin-deficient cells (deficient in mitochondrial ISC assembly) have 5-times the amount of GSH than WT cells? (Human Mol. Genet. 2008, 17:2790).***

Answer:

This paper (Auchere et al, 2008) shows that the yeast frataxin mutant (yfh1Δ) has a 2 to 5 fold decrease of total GSH levels, together with an increase of the GSSG/GSH ratio. It also shows that exogenous iron partially corrects GSH levels in mutants while decreasing it in wild type cells. The decreased GSH concentration does not seem to be linked to decreased synthesis or increased degradation or excretion. The authors linked the decrease of GSH to the prooxidant conditions created by the *YFH1* mutation. Irrespective of their interpretation, these data link GSH and iron metabolism at the level of the amount of GSH present in the cell, a link also established based on the yeast ATM1 mutant that carries an increase in total GSH with decrease of the GSH/GSSG ratio (Kispal et al, 1997). However, these data and ours do not contradict but rather complement each other. Cellular adaptation to iron or to an alteration in Fe-S biosynthesis/maturation might include a regulation of GSH cellular levels, the mechanism and purpose of which is not understood by us and needs further explorations.

Action:

We cited this and the Kispal paper in the discussion section, second paragraph, as link between iron and GSH homeostasis, the mechanism of which will need to be explored.

5. ***Figures are improperly labelled: where is Fig 6, E (page 9)? Also in page 9, Fig. 6D is Fig 7A, and Fig 6E of page 10 may be Fig. 7B, and 6F is 7C. A mess.***

Action:

We again apologize for the confusion brought about by errors in figure numbering. These errors were properly corrected.

In figure 7B, hr should be min, I guess.

Action: Yes, corrected.

There are many mistakes in the text while referring to figures and tables. Here are some: page 5 it says figure S3 instead of supplementary table S3;

Answer:

The reference is indeed to the supplementary fig. S3 that compares the 5 and 30 min HGT1 microarray data.

page 8: it should say Table S3 (but the Table is called S4, change it too).

Action: corrected. We also very carefully checked for any other errors.

6. ***Abstract, second line: 'To determine which of these functions...'. They have not established earlier in the text that there are several functions, only its role in ISC assembly. Re-write.***

Action:

We changed the wording of the first sentence to "GSH contributes to cellular thiol-redox control".

7. We are concerned about the growth and survival curves to GSH in HGT1 cells (Fig. 1B,C). What happens with the cells that do survive, are they the ones resuming growth after GSH depletion?

Answer:

Yes, this is what we think. A proportion of cells —which depend on the amount of GSH used— escape GSH toxicity, presumably because GSH is eventually degraded, as explained in results, first paragraph. As shown in fig. 1B, growth of HGT1 cells resumes after 5 to 10 hrs depending on the amount of GSH used, and growth resumption is concomitant with GSH levels correction (fig. 1D) (see also answer to next query).

No action

Why survival stays at 40% even at higher doses of added GSH? It would be nice to know the intracellular concentration of GSH at 200 μM (Table 1), since the growth and survival of cells at

this concentration is shown in Figure 1B and C. The authors should discuss why increasing the concentration from 50 μM to 200 μM GSH does not seem to result in decreased survival (Fig 1C) or why cells that do reach a similar OD after 25 h of treatment with increasing concentration of GSH (albeit with a small delay at 200 μM) then do not totally survive. It seems as GSH would have short term and long term effects.

Answer:

We previously showed that the Km of HGT1 for GSH is 50 µM (Bourbouloux et al, 2000). Therefore, uptake saturates above 50-100 µM GSH. Accordingly cell killing should be the same above these concentrations, which is what we see in fig. 1C. Accordingly also with higher amount of GSH, toxicity is delayed in time because of the delayed presence of GSH in the medium, and this is what is seen in the growth curves of fig. 1B. Accordingly also, the intracellular concentration should be the same with HGT1 cells incubated with GSH at either 100 or 200 µM, and this is also what the measures showed when performed after 1-hour incubation. The referee therefore made a good point by stating that GSH might have short and long terms effects, but these effects are kinetics and not related to GSH toxicity per se (higher GSH amounts do not increase the amplitude of GSH toxicity, but the length of time cells are exposed to this toxicity). We did not measure the kinetics of the GSH intracellular concentration upon incubating cells with 200 µM GSH, but we presume that the correction of GSH levels seen 2 hours after exposure to 100 µM GSH (fig. 1D) would be delayed by a couple of hours.

Action:

We added a short sentence in the result section, first paragraph, to clarify this point.

or why cells that do reach a similar OD after 25 h of treatment with increasing concentration of GSH (albeit with a small delay at 200 μM) then do not totally survive

Answer:

We were confused by the referee's comments: actually all cells that grew up to 25 hrs were fully alive.

8. Other differences between the transcriptome profiles of delta gsh1 and HGT1 cells (besides the already mentioned ER response and Yap1 dependent genes) is that the former does not (i) display major repression in ribosome biogenesis/translation (even though cells are dying), (ii) show altered carbohydrate metabolism. Explain.

Answer:

Repression of ribosome biogenesis/translation is a signature of the mammalian ER stress response, which is likely to be the case in the yeast ER stress response. However reported genome-wide analyses of the yeast ER stress response only focused on induced genes that are dependent on Ire1-Hac1, but not repressed ones (Travers et al, 2000). Our observation thus indicates the existence of a transcriptional repression of ribosome biogenesis/translation during ER stress, although this interpretation must also considerer the complex nature of GSH toxicity. Although a second genome-wide study of the yeast ER stress response did not report any translational response (Kimata et al, 2006), a genome-wide analysis of the *C. albicans* response to DTT/tunicamycin showed a transcriptional repression of translation and ribosome biogenesis genes (Wimalasena et al, 2008), which support our data. Microarray analysis of polysome-mRNA also identified transcript-specific translational regulation in *S. cerevisiae*, with translational repression of transcripts with functions in ribosomal biogenesis and assembly (Payne et al, 2008).

Both HGT1 and gsh1Δ cells displayed induction of carbohydrate metabolism as a presumable consequence of the defect in iron metabolism. However, we don't have a strong explanation for why the effect was much stronger in HGT1 —17 genes induced and 9 genes (mostly transporters) repressed—, than in gsh1Δ cells —only 4 genes induced. According to the current model described in the discussion, second paragraph, the atm1 mutant should phenocopy the genomic response seen upon GSH depletion as both gene product are likely to function in the mitochondrial export pathway: in fact, the carbohydrate metabolism response in the atm1 strain —8 genes induced— was not very different from the one observed in gsh1 Δ cells. The difference pointed out by the referee could possibly be due, at least partly, to the conflict between the ER stress and iron-starvation response in HGT1 cells.

Action:

We have incorporated in the text the reference relating the *C. albicans* data, as support of our assertion that translational repression is probably linked to the ER stress response.

9. Again regarding microarray data, in Figure 2 Legend, or either in the text, it should be described in more detail how the genes were assigned to functional categories, for instance which database was used.

Action:

We mentioned in fig. 2 legend which database we used for assignment of gene functional categories: http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl

10. More editing: Main text, page 6, lines 12 to 15: indicate which figure represents the stated affirmation;

Action:

The microarray data of ire1Δ overexpressing HGT1 was not shown. We now added a Venn diagram summarizing these data as supplementary fig. S4, C and D.

Figure 3D: indicate the GSH concentration at which the experiment was performed (in all cases, substitute the sentence 'at the indicated concentrations' by the actual concentration, is shorter and more useful);

Action:

The fig. 3 legend was edited as requested.

Figure legend 4F: not correctly explained;

Action:

The fig. 4 legend was edited as requested.

Figure 5B: indicate the concentrations of GSH, DTT and FeCl3 at which the experiments were performed;

Action:

The fig. 5 was edited as requested.

Figure 7/figure legend 7: indicate the h2o2 concentration at which the experiment was performed (it is only stated in results);

Action:

The figure was removed as suggested by the referee (see above).

supplementary table legends of Table S1 and Table S2, lines 1 and 2 in both cases, change the concentration of GSH from 50 mM to 50 μM;

Action:

Done. The change might have been caused by data conversion from Apple to PC, and might recur.

Figure legends of Supplementary figures S3 and S4, line 2 in both cases change the concentration of GSH from 50 mM to 50 μM;

Action:

Done.

Supplementary figure S7: include the line that represents the data of the strain ire1Δ (HGT1)

Answer:

The patch assay performed with the ire1Δ HGT1 strain was already shown in fig. 4H.

Action:

We did not follow the referee's recommendation, for not duplicating the same figure in the manuscript.

in Figure S1 A, the numbering of the Y axis should follow the same format.

Action:

Done.

11. In Table I, second column, the concentration of GSH at 100 μM should follow the same format as for the other lines, i.e. GSH (μm).

Action:

The table was edited by removing the word "GSH" in the second column.

12. Figure 6A does not seem to correspond to spot dilutions as described in the figure legends, but rather to plating a determined amount of cells onto plates with different GSH concentrations.
Action:

The referee is right. We added the amount of cells spotted in the legend of fig. 6A (2 10^6 cells).

2. REFEREE 2. We thank this referee for his/her thoughtful comments, to which we provide the following answers:

MAIN CRITICISM

-To unequivocally demonstrate that GSH toxic levels inhibit exclusively cytoplasmic Fe-S cluster maturation, w/o affecting mitochondrial physiology, yeast cells should be grown in a medium containing a respiratory carbon source (glycerol) rather then a fermentative carbon source (glucose).
Action:

We tested the toxicity of GSH in HGT1 cells grown on SC + glycerol (SG) plates (fig. Referee F, for referee-only. Data not included in the Peer Review Process File.) This experiment shows that GSH toxicity is not increased, but similar or very slightly decreased on a respiratory medium: there was still here some growth at 20 μM GSH, but not on SD (see fig. 1A). These data indicate that toxicity of high GSH levels is not contributed to by impairment of mitochondrial physiology (apart from the major microarrays genome remodeling that affect mitochondrial respiratory components).

-The authors demonstrate an increase in ROS production in cells grown in the presence of high GSH levels. Since Fe/S cluster is destabilized by ROS, the author should clarify the contribution of reactive oxygen species in the impairment in cytosolic Fe/S cluster. Is FET3 mRNA upregulated when cells are grown at high GSH levels, in the presence of a ROS scavenger?
Answer:

(i) We over expressed several ROS scavengers (the peroxiredoxins Tsa1, Tsa2 and Ahp1, cytosolic catalase —Ctt1— and cytochorme c peroxidase —Ccp1), but these transgenes did not affect GSH toxicity.

(ii) In HGT1 cells, H_2O_2 is presumably produced within the ER as a consequence of perpetuation of Ero1 cycling (paragraph 4 of results, fig. 4) (see response to referee 1, minor point 3), but does not seem to exit the ER: Neither Yap1 —the yeast H_2O_2 sensor— nor the peroxiredoxin Tsa1 —a thiol peroxidase that has a very high reactivity towards H_2O_2— became oxidized in HGT1 cells grown with GSH (data not shown).

(iii) More importantly, we showed (fig. 4F) that inactivating the Ire1 kinase (ire1Δ HGT1) blunted the generation of H_2O_2 triggered by GSH. However ire1D HGT1 were otherwise even more sensitive to GSH (fig. 4H) and retained the alteration in iron metabolism seen in HGT1 cells, as established by microarray analysis (new supplementary fig. S4, C and D).

(iv) In fact, growth of HGT1 cells on plates containing H_2O_2 decreased GSH toxicity for reasons that we have not yet understood (not shown). And this is not because GSH was converted to GSSG by oxidation, as GSSG is as toxic as GSH.

We believe these data strongly indicate that the H_2O_2 generation triggered by GSH cannot account for the iron phenotype of HGT1 cells.
Action:

We had already stated in paragraph 4 of results, last lane, that GSH toxicity is not caused by the H_2O_2 produced: "the impact of toxic levels of GSH on ER secretion contributes to its lethality, but this lethality is not caused by H_2O_2 production", and repeated this statement in discussion bottom of paragraph 4.

-In the second part of the manuscript, the authors describe the alteration of iron metabolism in conditions of GSH depletion. To better investigate how mitochondrial Fe/S cluster synthesis is impaired by the absence of GSH, yeast cells should also be grown in the presence of a respiratory carbon source.
Answer:

As shown initially by Sipos (Sipos et al, 2002) and here (supplementary fig. S6, moved to fig. 5), depletion of GSH did not impair activity of the mitochondrial Fe-S-based enzyme aconitase, in contrast to the cytosolic Fe-S-based enzyme Leu1. These data thus indicate that GSH depletion

affects cytosolic but not mitochondrial ISC assembly. Still, as recently shown in a paper published while this was in review (Ayer et al), GSH depletion leads to a loss of mitochondrial DNA (see below our response to the next comment).

Action:

As requested, we grew gsh1Δ cells on a respiratory medium (SD + glycerol), and observed that the GSH requirement was 25-50 time higher than on SD + glucose (fig. Referee G, for referee-only, data not included in the Peer Review Process File), a result consistent with the occurrence of a petite phenotype upon GSH depletion (Ayer et al; Kistler et al, 1986).

-Recently, a paper describing the critical role of GSH in maintenance of mtDNA and iron homeostasis has been published (Ayer et al., Free Radical Biology and Medicine, doi: 10.1016/j.freeradbiomed.2010.09.023). The results presented by the authors must be re-evaluated, and more emphasis should be given to the novel implications with respect to this publication.

Answer:

We thank the referee for his question. This study (Ayer et al) rigorously quantified the respiratory incompetency occurring upon GSH depletion and showed it is caused by mitochondrial DNA (mtDNA) loss, also elegantly showing that mtDNA loss is a direct function of the iron accumulated in gsh1Δ cells as corrected by iron deprivation.

The Lill's group showed that the excess iron of GSH-depleted cells is contained within mitochondria that carry more than 40 time iron than wild type (Sipos et al, 2002), as most of the mutants in both mitochondrial Fe-S assembly and mitochondrial export systems (ex: ATM1) (Kispal et al, 1997; Kispal et al, 1999). Why iron accumulates in mitochondria in these mutants is not understood.

Accordingly, mtDNA loss and the ensuing respiratory incompetency of GSH-depleted cells appear as a late consequence of the prime defect triggered by this condition, the defect in cytosolic Fe-S assembly (as proposed by Lill and coworkers, and here) and of the resulting mitochondrial iron accumulation, which alter DNA, presumably through Fenton chemistry-mediated ROS production (as suggested by Ayer).

Therefore, the study of Ayer and ours focus on a different aspect of GSH depletion, the petite phenotype/mtDNA loss and what causes GSH auxotrophy, respectively, and do not contradict each other. However, together these study show that paradoxically iron is both toxic for mtDNA (Ayer et al) and can partially rescue growth, as shown here. The improved iron growth rescue under anaerobiosis (new fig. 5D) and the observation that under aerobiosis iron > 100 μM was much less efficient (not shown) are consistent with the opposite effects of exogenous iron on GSH-depleted cells. Such opposite effects of iron may also explain why Ayer could not totally correct the respiratory incompetency of GSH-depleted cells by iron deprivation since, such regimen probably has a counter effect on cell viability (since as shown here iron improves viability, lack of it should decrease it). To explain iron only partial correction, these authors claimed the presence of an iron-independent mechanism of mtDNA loss; however, they did not check the amount of iron remaining in mitochondria after iron deprivation.

Lastly, the study of Ayers also supports ours by suggesting that loss of mtDNA in GSH depleted cells —which we explained above is a consequence of defective cytosolic Fe-S maturation— is independent of the change of the GSH redox potential occurring in these cells (more oxidized), which support our claim that the function of GSH in iron metabolism is not a redox one.

Action:

We retested the effect of iron on GSH-depleted cells by considering that if cells were already petite, toxicity of iron on mtDNA should extinguish and rescue should therefore be much more potent. As predicted, using a gsh1Δ petite strain grown up to 9 divisions in medium lacking GSH (drastic GSH depletion), rescue by iron was now much more potent. Furthermore as also predicted, excess iron was now not toxic, but on the contrary further improved growth (better rescue at 500 than at 100 μM FeCl3), and rescue was now similar under anaerobiosis and aerobiosis. These new data have been included in the paper as fig. 5, D and E, and fig. 5 C is now included as new supplementary fig. S7B. The result section has been modified accordingly and now cites the Ayer paper. The discussion (paragraph 2) has been edited accordingly.

-The dual role of GSH in ISC maturation and redox buffering poses an interesting evolutionary problem. The authors might wish to embark in a few speculations concerning what came first.

Answer:

Fe-S clusters are evolutionary ancient prosthetic groups required to sustain fundamental life processes. Similarly the use of cysteine-based redox chemistry in enzymatic reaction and of and

disulfide bond formation for establishing covalent linkages is also very ancient evolutionarily. Surprisingly in contrast to eukaryotes, GSH is not essential in most prokaryotes, except cyanobacteria, and absent in others as in *B. subtilis*. Further, data indicating a role of GSH in Fe-S assembly or more generally iron metabolism in prokaryotes are to our knowledge absent. These data would tend to indicate that the GSH redox function might have predated its function in iron metabolism. However, prokaryotes paradoxically possess monothiol glutaredoxin enzymes, some of which as *E. coli* Grx4, have been shown to form GSH-ligated Fe-S clusters by dimerization. Surprisingly again, grx4 but not gshA is an essential gene in *E. coli*. It seem, therefore that one must first sort out the still non described but probably existing functions of GSH in prokaryotic iron metabolism, before one is able to rigorously decipher this question.

MINOR POINTS (REFEREE 2)

-In many sections, the writing can be improved considerably. As presented, it is not linear enough to attract a wider readership.

Action:

We revised the writing style, as best as we could, considering the restriction imposed by the words limit of the journal, and we hope the changes introduced will satisfy the referee's expectations.

REFEREE 3. We thank this referee for his/her thoughtful comments, to which we provide the following answers:

Overall, the results in this manuscript would be of interest to a general reader were they to provide mechanistic insight into the role of GSH in iron metabolism and/or its redox function.

Answer:

This study provides a system view of GSH physiological role, rather than a molecular description of it. This system view led us propose a novel model of GSH physiological role that reconsiders its importance in cytosolic thiol-redox control, while emphasizing its crucial role in iron metabolism and explaining its essential requirement. Accordingly, we think that although of crucial importance, mechanistic insights into GSH molecular functions in iron metabolism fall beyond the scope of this study. We nevertheless explored and provide here several molecular endpoints of the consequence of the presence of GSH toxic and depletion throughout the paper that use genetic, biochemical and enzymatic approaches. Included also are novel experiments that have been requested by referee 1 and 2.

While the authors provide correlative results on GSH accumulation and activity of mitochondrial and cytoplasmic Fe-S proteins, experiments that provide more mechanistic information are needed to increase insight into this important question.

Answer:

We agree with the importance of this question, but again as stated above we believe this question fall beyond the scope of this study (please see the response to point 1).

Addition of iron rapidly reduced the high FET3 expression of GSH deficient yeast and partially rescued growth of gsh1-delta cells in media lacking GSH. The authors should be cautioned that FET3 mRNA levels may not reflect only Aft1 activity but is also a reflection of mRNA degradation, which for FET3 mRNA is rapid in the presence of excess iron. The use of a reporter gene linked to the FET3 promoter would help the authors to sort out such questions.

Answer:

This is an interesting point; we have unfortunately not been able to monitor FET3 expression upon addition of iron in gsh1Δ cells using a FET3-reporter gene. However, the data of the effects of iron on FET3 expression and growth in gsh1Δ cells are reciprocally supportive and the growth rescue of GSH-depleted cells by iron is dominant over the effect of iron on FET3 expression for the message of the paper. We have actually repeated and slightly extended the later data (please, see answer to referee 2, point 4), which further describes the growth rescue of GSH-depleted cells by iron. Nevertheless, as we could not verify experimentally the referee's point, we decided to downplay the results of the effect of iron on FET3 expression, saying that such a decrease could also be caused by mRNA degradation. However, we could not find any reference to support this claim, but an unpublished observation in a review article (Philpott & Protchenko, 2008).

The ability of DTT to rescue growth of gsh1 yeast further complicates interpretation of these results. Prior investigations have concluded that this ability of DTT to rescue gsh1 yeast demonstrates a thiol-redox function of GSH. Again, without mechanistic insight, it is not possible to distinguish between these two possibilities, although the authors do clearly show that much less GSH is required for cell viability than for general redox control.

Answer:

Please see the answer to referee 1, major point 2. The best indication that the function of GSH in iron metabolism is not a redox one (we think, we understood this was the referee's query) is the ability of iron to potently rescue growth of GSH-depleted cells, and this much better than DTT (see the new fig. 5, D and E). As comparison, in *E. coli* in which GSH has no known function in iron metabolism, DTT rescues growth of a strain lacking thioredoxin reductase and glutathione reductase (trxB gor) (Gon et al, 2006; Ortenberg et al, 2004).

ADDITIONAL POINTS: (REFEREE 3)

1. The addition of iron to yeast with toxic levels of glutathione did not protect. Does the reduction of iron have a protective effect or is it detrimental?

Answer:

We tested desferroxamine, but this did not change the toxicity level of GSH on HGT1 cells. However, it must be considered that the toxic impact of GSH on secretion is dominant over the one on iron metabolism, as indicates a partial remediation of GSH toxicity by the thiol oxidant diamide (Igbaria et al., follow up study in preparation; diamide decreases the reductive stress imposed on protein oxidative folding in a narrow range of concentrations), and the exacerbation of GSH toxicity in the ireD strain (fig. 4H). Accordingly, one may only see effects worsening (as excess iron) but not decreasing the GSH toxic impact on iron metabolism, as such effect would be offset by the remaining toxic impact of GSH on secretion that is dominant (see response to referee 1, point 6). Thus, even if desferroxamine could indeed decrease the iron component of GSH toxicity, we would not see it.

Action:

We have cited the desferroxamine experiment as data not shown in the result section and in discussion (paragraph 4)(see response to referee 1).

2. In figure 3D was 100 and 250 millimolar FeCl3 really used for this experiment?

Answer:

We are confused by this question as we read micromolar and not millimolar amounts of $FeCl_3$ in both the figure and its legend. Conversions from Apple to PC might have modified founts reading.

3. Page 10, line 7: (fig. 6F) should read (fig. 7C).

Answer:

We are sorry for the confusion brought by mislabeled figures. Corrected.

4. It would be helpful to add wild type plus GSH to Table I.

Excess GSH does not modify GSH intracellular levels in wild type cells. We thus thought this line was unnecessary.

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2nd Editorial Decision 18 January 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now all positive about publication of your paper. Still, referees 1 and 3 are not yet fully convinced that the evidence presented for your main conclusion that the main physiological role of GSH is its involvement in ISC assembly (rather than a general redox function) is strong enough and therefore suggest addressing a few points further (see below). I would therefore like to ask you to deal with or respond to the issues raised in an adequate manner in an amended version of the manuscript.

Furthermore, there are two remaining editorial issues that need further attention. Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes and yeast growth assays that are assembled from different plates. While cropping and pasting may be considered acceptable practices in some cases there needs to be a proper indication as well as an explanation in the figure legend in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or explanation to ask for the original scans. In the case of the present submission there are a number of panels that do not appear to fully meet these requirements: figures 6A and 6B; S5D. I therefore like to kindly ask you to include an explanation in the figure legends how the panels were assembled in the amended version of the manuscript. Please be reminded that according to our editorial policies we also need to see the original scans for the panels in question. Furthermore, I would like to ask you to include the statistical details into the legends of figures 4A, B, E; 5A, C; 7; S5D, E and the table.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

First of all, I would like to thank the authors for the response to our previous suggestions, and for the changes introduced in the manuscript, many of which have improved it especially regarding clarity, format, typos (figure numbering especially) and general reading.

As I already indicated in my first revision, I had the impression that the authors had written an integrative report or a review, rather than an original manuscript, because most of their individual "findings" had already been reported, in one way or the other. I was expecting the new material to emphasize the very few new outcomes of their research. It was already published that cells lacking GSH have problems regarding thiol control, eletrophilic compounds scavenging and iron sulfur cluster (ISC) assembly. The nicest contribution of this manuscript would be to unambiguously demonstrate that the main and essential role of GSH in yeast is in ISC assembly, since it helps Grx3 (or 4) to coordinate an ISC and transfer it to the ISC cytosolic pathway. To fully demonstrate so, proper complementation of the defects of the delta gsh1 strain with Fe and not with DTT should be done.

Many of our previous concerns/doubts/questions have been answered and/or discussed, and we fully or partially agree with most. We are left with the following problems:

Major points

1) We are very much surprised (and did not realise before, our mistake) that the authors did not include, at least in their 2nd version, a relevant manuscript regarding the topic of interest: Muhlenhoff et al., Cell Metabolism 2010, 12:373. The role of Grx3/4 in iron sensing and ISC assembly is dissected in this manuscript. After reading the author's response to the reviewers, it is very obvious that GSH binding the ISC of Grx3/4 is the main role of GSH in yeast, which should explain the redox-independent role of the tripeptide. And this could be inferred from the above manuscript, since Grx3/Grx4 are also essential due to this reason, and need GSH to bind their ISC. In the Cell Metabolism manuscript, the authors even tested whether GSH binds to Grx3/4 in vivo (it had only been done before in vitro), by using (and I quote) the "GSH synthesis-deficient mutant gsh1Δ that can be depleted for GSH upon growth in media lacking GSH. Although significant amounts of 55Fe were bound to Grx4-Myc in the presence of exogenously added GSH, only background levels of 55Fe were found in GSH deprived cells (Figure 5F)". More than that, these and many other experiments force them to conclude that GSH performs a dual role in cellular iron metabolism. As part of the Grx3/4 complex, it is central for intracellular iron delivery and sensing, and as a component of the ISC export machinery. Kumar et al. should include this reference, and comment it.

While Muhlenhoff et al. describe this essential role of Grx3/4 in iron trafficking and sensing, they claim that cells lacking this proteins had impaired mitochondrial activities such as Aco1 (not only cytosolic FeS enzymes such as Leu1). How do you reconcile that GSH is only required for Leu1, but not for Aco1, if Grx3/4 are?

2) The main contribution of the Kumar et al. manuscript, or that is what we believe, is to clarify pre-existing (and probably misleading) reports regarding complementation of delta gsh1 cells, which was claimed to occur with DTT (Sipos et al, 2003, Grant et al, 1996, for instance). The need of only 0.5 uM GSH being sufficient to complement delta gsh1 is very convincing now with the aerobic and anaerobic plates (Fig. 6A and B); authors should run exactly the same experiment (at least with WT and delta gsh1, but optimally using the same strains as Fig. 6AB) with DTT and iron (current figures 5C, 5D and 5E are still very preliminary, experimentally defective and misleading; for instance, GSH 1 mM in Fig. 5C is too high; colony sizes are different in 5D, and growth recovery upon iron in anaerobiosis is limited; the use of petit strains is confusing and the only one used for DTT (it is not clear whether wt strains behave similarly), etc). I really do not understand why the authors have

not made the effort to present proper complementation figures, which were requested by several reviewers.

Minor points

1) To clarify the HGT+GSH model, we suggested over-expression of glutaredoxins, and it has not worked. Alternatives hypothesis or alternative experiments should have been tried. 30 mM glutathione (as shown in Table 1) should required huge amounts of Grx3/4 to avoid titration of the glutaredoxins, and proper concentrations-dependent experiments have not been performed (although at least a partial improvement have been reported at low GSH concentrations; suppl. Fig. S5E).

2) Upon request, authors have measured concentration of GSH in delta trr1 strain, and edited the former Table 1, and we appreciate it. However, they say all over the text that the levels of GSH are 2-3 fold in delta trr1 cells respect to WT, probably according to previous reports, but they barely report 1.5-fold increase. Correct or explain.

3) We also requested the authors to strengthen their hypothesis (GSH being essential only for its role in ISC pathway) by showing NEW experiments (not the 2002 reported effect of atm1 deletion of single delta gsh1 strain, performed with strikes and not spots; Sipos et al.) reporting genetic interactions with other CIA components (either by deletion or over-expression).

Referee #2 (Remarks to the Author):

The authors have adequately answered to most of the criticisms raised to the original version of their manuscript. The revised version is now suitable for publication in the EMBO J.

Referee #3 (Remarks to the Author):

The revised manuscript from Kumar et al. has been improved. The authors have extensively addressed the reviewer's comments, and have strengthened their argument that the essential role of glutathione is related to a function in cellular iron metabolism. My concern is that the manuscript remains mainly phenomenological, however. The authors posit that high, toxic and low, deficient GSH both induce the iron regulon because of effects on cytosolic Fe-S cluster assembly. A major mechanistic question is whether this essential function of GSH relates to a direct role in Fe-S cluster biogenesis or in some other aspect of iron metabolism. New data in the manuscript show very convincingly that addition of iron to GSH depleted cells restores some growth, consistent with the notion that an essential process of iron metabolism is defective in GSH depleted cells. In fact, in anaerobiosis 500 micromolar FeCl3 appears to restore growth as well as 1 mM GSH, which suggests that essential processes had been restored. However, effects on Fe-S cluster assembly were not investigated. FET3 expression was followed for some conditions, but this is not a direct measure of cytosolic cluster biogenesis. The authors should show whether iron as well as the minimum concentrations of GSH required for viability could restore Fe-S cluster assembly in GSH depleted cells, which could be done by looking at Leu1 enzymatic activity. Whether iron restores cluster assembly or not, this analysis would provide important mechanistic insight into the essential role of GSH in iron metabolism, which I believe would increase interest in this study.

Additional points:

1. The statement on page 5, first paragraph, line 5 is confusing. First, it is not clear that there is a cell killing dose effect below 100 micromolar GSH since there is only one concentration lower than this shown in Fig. 1C. Second, I would expect a similar longer lag before growth resumption for cells placed into media with 100 micromolar GSH given that this concentration is well above the Km of HGT1. The authors need to modify the sentence to communicate the results more appropriately.

2. If 50% of cells are killed by excess GSH within 1 hour, then how do the authors explain near normal activity for Aco1 and other proteins not affected by GSH shown in Fig. 3?

3. The authors should remove the reference to the experiment using desferroxamine to deplete cells of iron (p.6, section 3, line 11 in paragraph). The genetic experiments using the fet3 and aft1/aft2 strains are sufficient, and yeast can utilize desferroxamine iron making it dubious that it was an effective chelator. Dipyridyl or bathophenathroline are better chelators for this purpose.

4. Hasset et al. (JBC, 273: 7628-7636) showed that FET3 mRNA was lowered by >90% within 10 min after iron addition, indicating very rapid mRNA degradation in the presence of iron. This potentially complicates the interpretation of the iron effect on FET3 mRNA level in GSH-deficient cells.

2nd Revision - authors' response 22 February 2011

Response to reviewers

The manuscript has been edited according to the referee's requests. We have also edited the discussion in light of the new data of fig. 5, D and of a new reference. We also made stylistic changes in the text that are also highlighted, for the most part.

In the figures, we introduced the following changes:
- Fig. 5, D indicates the partial correction by iron of the Leu1 activity defect of GSH-depleted cells.
- Supplementary fig. S7, C indicates synthetic lethality between *GSH1*, *GRX3* and *GRX4*.

Referee #1

We thank this referee for her/his appreciation and comments, to which we provide the following answers:

Major points

1) *We are very much surprised (and did not realise before, our mistake) that the authors did not include, at least in their 2nd version, a relevant manuscript regarding the topic of interest: Muhlenhoff et al., Cell Metabolism 2010, 12:373.*

Answer:

We understand the referee's point: this paper came out after we submitted ours; although we were aware of it, we did not address it since referees did not request. This paper shows that GSH is required for iron loading on Grx3/4. This importantly suggests that GSH assembles a Fe-S cluster with Grx3/4, but then focuses on GRX3/4 and does not reveal more on the role of GSH in iron metabolism. In fact it raises difficult questions with regards to GSH function (see below).

Action:

At the referee's request we have now cited this paper in the discussion, second paragraph, which required editing some parts of the discussion.

While Muhlenhoff et al. describe this essential role of Grx3/4 in iron trafficking and sensing, they claim that cells lacking this proteins had impaired mitochondrial activities such as Aco1 (not only cytosolic FeS enzymes such as Leu1). How do you reconcile that GSH is only required for Leu1, but not for Aco1, if Grx3/4 are?

Answer:

This is a difficult question raised by the Muhlenhoff study. There is another important difference between the phenotypes of GSH and Grx3/4-depleted cells: the former accumulate iron in mitochondria, while the latter does not, which has been taken by these authors to indicate the mitochondrial iron delivery function of Grx3/4. Thus, if Grx3/4 have functions not carried by GSH, then the role of the GSH-Grx3/4 Fe-S cluster becomes a puzzle. We believe, that in fact GSH has wider function in iron metabolism, but these cannot be phenotypically revealed, because of the unavoidable incomplete nature of GSH depletion: distal GSH-dependent functions might be altered first, causing cell death before proximal functions reveal. For instance, involvement of GSH in mitochondrial ISC assembly is likely but cannot be tested at the present time due to our inability to evaluate the extent of GSH depletion in mitochondria. Much work will be needed to elucidate the functions of GSH in iron metabolism and its partnership with Grx3/4.

The main contribution of the Kumar et al. manuscript, or that is what we believe, is to clarify pre-existing (and probably misleading) reports regarding complementation of delta gsh1 cells, which was claimed to occur with DTT

Answer:

We suggest the referee not to minimize the important part of the study indicating that GSH has an ancillary role in cytoplasmic thiol-redox control that challenges the concept of GSH as a thiol-redox buffer in the cytosol.

2) *The need of only 0.5 uM GSH being sufficient to complement delta gsh1 is very convincing now with the aerobic and anaerobic plates (Fig. 6A and B); authors should run exactly the same experiment (at least with WT and delta gsh1, but optimally using the same strains as Fig. 6AB) with DTT and iron (current figures 5C, 5D and 5E are still very preliminary, experimentally defective and misleading; for instance, GSH 1 mM in Fig. 5C is too high; colony sizes are different in 5D, and growth recovery upon iron in anaerobiosis is limited; the use of petit strains is confusing and the only one used for DTT (it is not clear whether wt strains behave similarly), etc). I really do not understand why the authors have not made the effort to present proper complementation figures, which were requested by several reviewers.*

Answer:

We are confused by this comment: this referee (nor any other in fact) never asked we repeat the iron rescue experiment, but instead suggested in his major point 3 that *"Combination of extracellular iron and GSH should be synergistic on growth (Fig. 5A)?*" which we answered by providing figure C for referee-only (data not included in the Peer Review Process File).

Although this was not asked, we made the effort of repeating this experiment, because we thought iron might have paradoxical negative (loss of mtDNA) and positive (growth rescue) effects.

With regards to the news issues raised by this experiment:

a) Fig. 5 and fig. 6 used exactly the same strains; they only differ by the petite phenotype (see below answer e).

b) In fig. 5E, GSH is used at the concentration of 1 mM to serve as an appropriate WT growth control (this is the GSH amount routinely used to maintain this strain. Lower concentrations would have been inappropriate for a proper WT growth control.

c) The colony size difference between aerobiosis and anaerobiosis is unavoidable, due to the presence of ergosterol in the plates used for anaerobic growth (molecular oxygen is needed to synthesize ergosterol in yeast), which increases the surface tension of the plate and causes increased spreading of cell culture drops.

d) The limited iron growth rescue is linked to the partial nature of rescue: iron-rescued cells do not grow again when re-streaked in plates containing iron and no GSH.

e) *Use of petite is confusing:* as previously explained, mtDNA loss (petite phenotype) is an unavoidable consequence of GSH depletion {Kistler, 1986 #95}, and is increased by iron {Ayer, 2010 #88}. Initial iron rescue experiments were performed with GSH-depleted cells that were not homogeneous with regard to mtDNA status; we thus repeated the experiment with a homogeneous petite cell population, and by considering that iron might have an adverse effect on growth rate by increasing mtDNA loss, as much as beneficial.

Action:

The experiment shown in fig. 5 is representative of several other experiments. As this is the best we can do, we don't see how repeating the experiment will improve it. We edited the text to clarify the issue of the petite cells phenotype. Please, see also the new experiment showing the rescue of Leu1 activity by iron in response to referee 3, first point, which should address the concern raised here by the referee.

Minor points

1) *To clarify the HGT+GSH model, we suggested over-expression of glutaredoxins, and it has not worked. Alternatives hypothesis or alternative experiments should have been tried. 30 mM glutathione (as shown in Table 1) should required huge amounts of Grx3/4 to avoid titration of the glutaredoxins, and proper concentrations-dependent experiments have not been performed (although at least a partial improvement have been reported at low GSH concentrations; suppl. Fig. S5E).*

Answer:

We fully agree with the referee, and it is for this very reason that we tested the growth of HGT1 cells overexpressing *GRX4* in the presence of low amounts of GSH from 5 to 25 micromolar (supplementary fig. S5, F); we did not see however any growth improvement, even at these very low GSH concentrations.

2) Upon request, authors have measured concentration of GSH in delta trr1 strain, and edited the former Table 1, and we appreciate it. However, they say all over the text that the levels of GSH are 2-3 fold in delta trr1 cells respect to WT, probably according to previous reports, but they barely report 1.5-fold increase. Correct or explain.

Answer:

Considering total GSH levels (GSH + GSSG), our measures give in *trr1Δ* = 12.8 mM/cell and in WT = 7.41 mM/cell. Accordingly, the mutant strain has a 1.73 fold increase. Published data arrive at a 3.25 fold increase in *trr1Δ*, compared to WT.

Action:

We edited the text, changing 2 to 3 fold by 1.5 to 3 fold increase.

3) We also requested the authors to strengthen their hypothesis (GSH being essential only for its role in ISC pathway) by showing NEW experiments (not the 2002 reported effect of atm1 deletion of single delta gsh1 strain, performed with strikes and not spots; Sipos et al.) reporting genetic interactions with other CIA components (either by deletion or over-expression).

Answer:

(i) We also requested the authors to strengthen their hypothesis (GSH being essential only for its role in ISC pathway): We suggest the referee to consider the new experiment to referee 3, first point, showing the partial correction of Leu1 activity by iron, which also addresses this referee's point.

(ii) Is delta gsh1 synthetic lethal with mutations in genes coding for mitochondrial or cytosolic ISC? We now show (see below) that *GRX3* and *GRX4* are synthetic lethal with *GSH1*.

(iii) o/e of ISC machinery components can suppress the defects of delta gsh1 cells? As mentioned, we conducted several genetic screens for genes whose overexpression would suppress the GSH auxotrophy of the *gsh1Δ* strain but we failed identifying any such genes (Spector D, Ph.D. thesis, 2001, and current unpublished data). We also conducted a genetic selection for extragenic suppressors of the GSH auxotrophy of *gsh1Δ D* cells but did not identify any true suppressors (Spector et al, 2001). As both screens were saturating, we conclude that genes whose overexpression or mutation could suppress the inviability of GSH-depleted cells do not exist.

Action:

GRX3 and *GRX4* are required for extra-mitochondrial Fe-S assembly, and their deletion is not lethal in the S288C background {Ojeda, 2006 #16; Pujol-Carrion, 2006 #17; Muhlenhoff, #90}. As synthetic lethality between these and *GSH1* would be more easily tested and interpreted than between *GSH1* and genes encoding essential CIA components, we constructed a strain lacking *GRX3*, *GRX4* and *GSH1* (*gsh1Δgrx3Δgrx4Δ*): this strain has a slightly higher GSH requirement than *gsh1D*, which indicates synthetic lethality. This experiment has been added as supplementary fig. S7, C, and is mentioned in the discussion section, second paragraph.

Referee #2 (Remarks to the Author):

The authors have adequately answered to most of the criticisms raised to the original version of their manuscript. The revised version is now suitable for publication in the EMBO J.

We thank this referee for her/his appreciation.

Referee #3 (Remarks to the Author):

We thanks this referee for her/his appreciation and comments, to which we provide the following answers:

My concern is that the manuscript remains mainly phenomenological, however. The authors posit that high, toxic and low, deficient GSH both induce the iron regulon because of effects on cytosolic Fe-S cluster assembly. A major mechanistic question is whether this essential function of GSH relates to a direct role in Fe-S cluster biogenesis or in some other aspect of iron metabolism.

Answer:

Our study provides a system view of GSH physiology that addresses its two sides, thiol-redox and iron. An important part establishes that GSH thiol-redox control function is negligible, except in the secretory pathway, which together with the *trr1Δ* strain model, showing that GSH

cannot sustain by itself the redox load of the cell, lead us to conclude that GSH serves at best as feedback of thioredoxin in cytoplasmic thiol-redox metabolism; this is a major aspect of the paper that should not be minimized, as challenging the very notion of GSH as a cellular thiol-redox buffer, and also disqualifying a redox function as causing the GSH requirement for viability. The other part indicates the physiologic importance of GSH in iron metabolism, which together with the iron growth —and now Leu1 activity (see below)— rescue of GSH-depleted cells lead us to suggest, that this (still undefined) function underlies the GSH viability requirement. Lastly we show that an increased redox load on GSH impinges on iron metabolism, which is also we think, a significant concept. As a system view, the study remains necessarily "*phenomenological*"; it does not address the molecular function of GSH in iron metabolism, which is not trivial and will require tremendous effort and time to be solved, but provide instead a novel cellular model of GSH functions.

However, effects on Fe-S cluster assembly were not investigated. FET3 expression was followed for some conditions, but this is not a direct measure of cytosolic cluster biogenesis. The authors should show whether iron as well as the minimum concentrations of GSH required for viability could restore Fe-S cluster assembly in GSH depleted cells, which could be done by looking at Leu1 enzymatic activity. Whether iron restores cluster assembly or not, this analysis would provide important mechanistic insight into the essential role of GSH in iron metabolism, which I believe would increase interest in this study.

Answer:

We understand the validity of this point, but also notice this is a novel request of this referee.

Action:

We performed this experiment: a one-hour exposure of six-divisions GSH-depleted cells to $FeCl_3$ (300 micromolar) increases Leu1 activity from levels that are undetectable to more than half of those reached upon cell exposure to 1 μM GSH. This result is from three independent experiments, two of them performed on three independent replicate cell cultures, and one on only one culture sample, all showing the same result. The experiment is added as fig. 5, D, and described in the result section, paragraph 5 and is discussed in the second paragraph of discussion. Our interpretation is that GSH, which is known capable of chelating iron, might provide the metal for cluster assembly on Grx3/4 or perhaps directly on apoenzymes in the CIA, which would then suggest an iron delivery function for GSH.

Additional points:

1. The statement on page 5, first paragraph, line 5 is confusing. First, it is not clear that there is a cell killing dose effect below 100 micromolar GSH since there is only one concentration lower than this shown in Fig. 1C. Second, I would expect a similar longer lag before growth resumption for cells placed into media with 100 micromolar GSH given that this concentration is well above the Km of HGT1. The authors need to modify the sentence to communicate the results more appropriately.

Answer:

If we understood the referee's question, she/he thinks that 100 and 200 micromolar GSH should give a similar lag before growth resumption, which is not the case: thus at 200 micromolar, GSH will remain longer in the medium than at 100, and hence will have a more prolonged effect on growth inhibition.

Action:

We edited the sentence according to the above explanation.

2. *If 50% of cells are killed by excess GSH within 1 hour, then how do the authors explain near normal activity for Aco1 and other proteins not affected by GSH shown in Fig. 3?*

Answer:

This is a fair point that was also alluded to by referee 2. Our preliminary experiments, using a mitochondria matrix redox biosensor equilibrating with the GSH redox couple, indicate that in HGT1 cells, the increased GSH levels are apparently excluded from this compartment.

3. The authors should remove the reference to the experiment using desferroxamine to deplete cells of iron (p.6, section 3, line 11 in paragraph). The genetic experiments using the fet3 and aft1/aft2 strains are sufficient, and yeast can utilize desferroxamine iron making it dubious that it was an effective chelator. Dipyridyl or bathophenathroline are better chelators for this purpose.

Answer:

Done

4. Hasset et al. (JBC, 273: 7628-7636) showed that FET3 mRNA was lowered by >90% within 10 min after iron addition, indicating very rapid mRNA degradation in the presence of iron. This potentially complicates the interpretation of the iron effect on FET3 mRNA level in GSH

Answer:

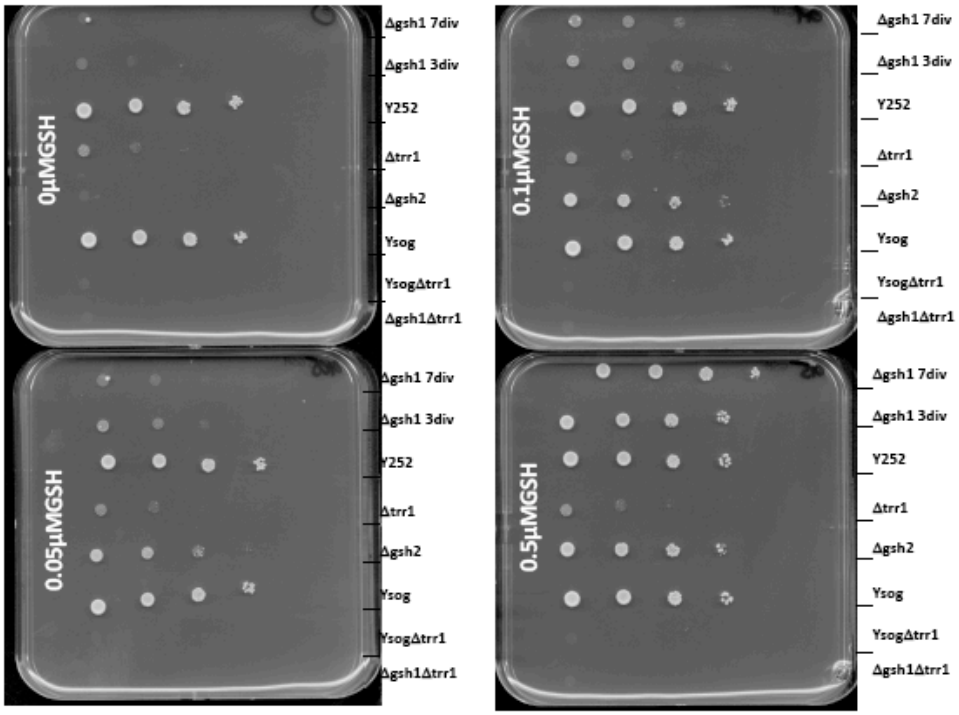
We thank the referee for highlighting this data, and note the validity of its remark. The iron rescue of Leu1 activity should now address this concern of the referee. We thus further downplayed the *FET3* mRNA levels data in the result section, citing the reference of Hassett et al.

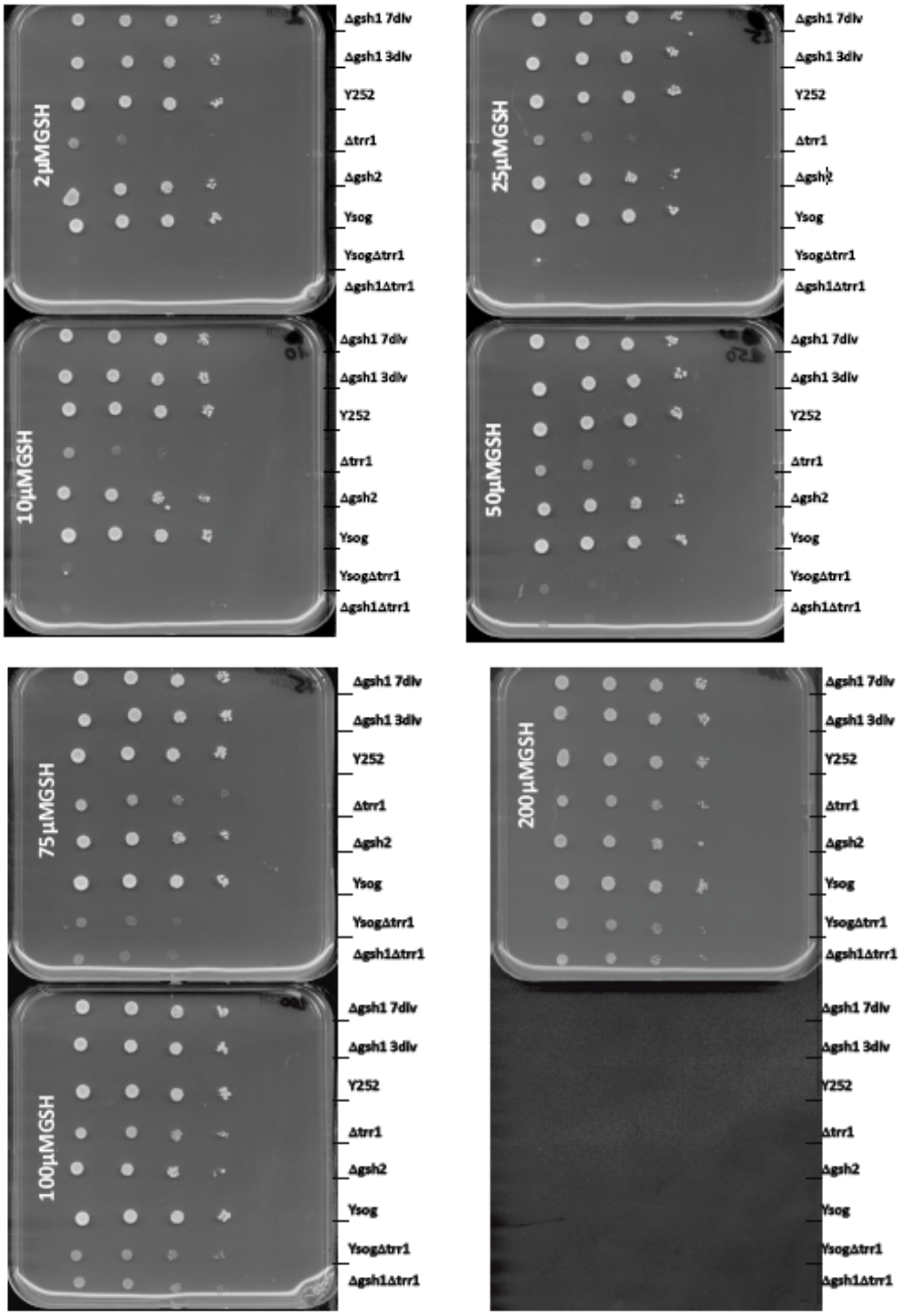
We addressed the editorial issues regarding how figures were assembled, and provide the requested full figures and the statistical details for those that were missing.

Response to editor

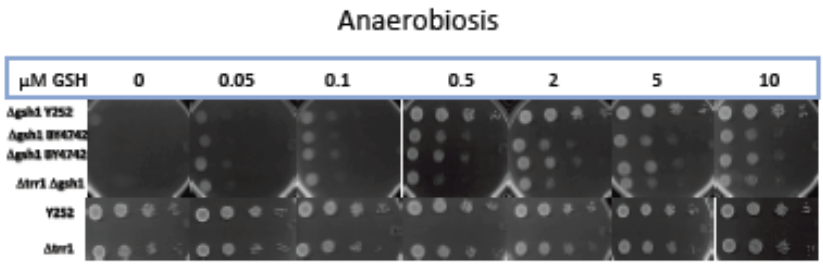
Here are the requested documents as Pdf files: fig. 6A, 6B, and supplementary S5D full scans.

Original scans Figure 6A

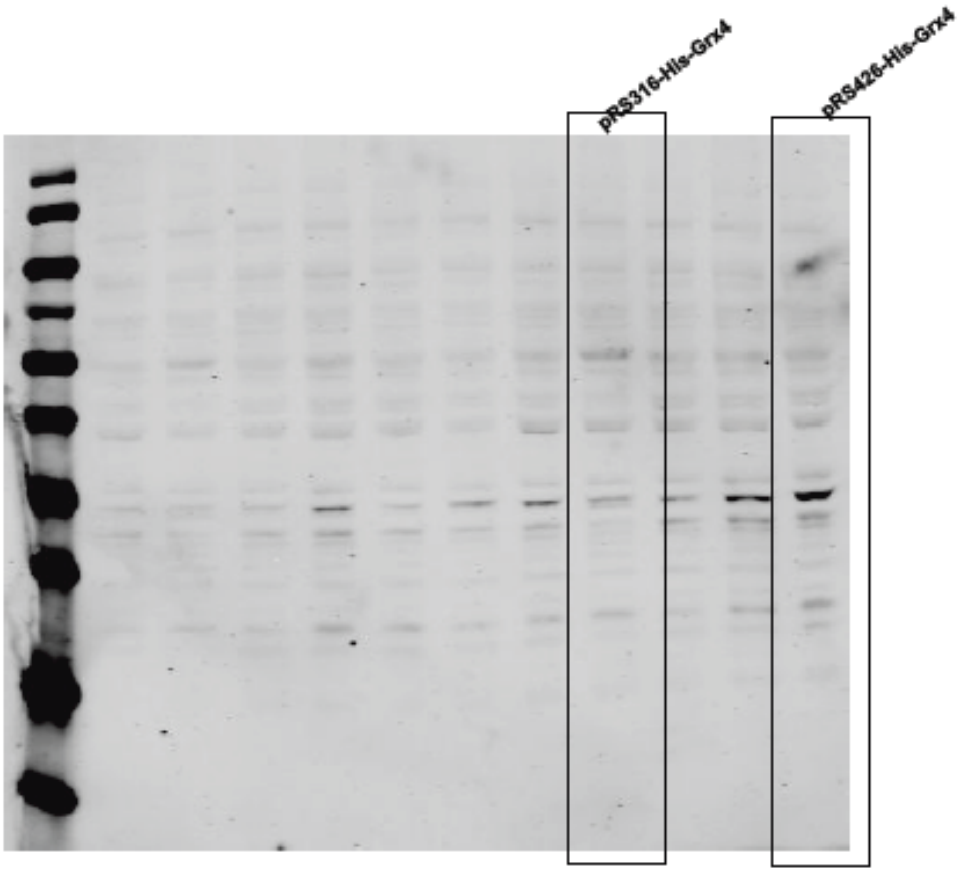




Original scan Figure 6B



Original scan Figure
S5D



3rd Editorial Decision 16 March 2011

Thank you for sending us your re-revised manuscript. Referee 1 has now seen it again, and now supports publication. Still, he/she raises a number of minor points that you may wish to address. In addition, according to our guidelines, I need to ask you to include a paragraph with the authors' contributions below the acknowledgement section. Please send us the final modified version of the manuscript text via e-mail. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee 1

We have read and reviewed the 3rd version of the manuscript entitled "Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control", submitted by Michel Toledano and colleagues for publication in the EMBO Journal. From the second to the third version of this manuscript, the main changes regarding our last suggestions refer to the inclusion of the Muhlenhoff et al. reference (Cell Metabolism 2010, 12:373), which forced the authors to considerably change the discussion, and the new Fig. 5D. Supplementary Fig. 7C is also new, and is related to the above mentioned manuscript.

We do not minimize the importance of what is stated in this manuscript, which is the fact that GSH is essential for iron metabolism (probably, ISC assembly) and not due to its redox buffer role. While trying to improve the manuscript, we raised some conceptual concerns (most of which have been nicely answered by the authors) and some experimental ones (mostly regarding concentrations and figure presentation). We believe that finding out the exact role of GSH in ISC assembly (cytosolic, mitochondrial or both) is not the goal of this manuscript, and we do not dare to ask for new experiments. We will have to accept that the authors feel unable to improve their current figures with new, experimentally nicer ones. Things that have been left unattended are indicated below (minor points).

Minor points

1) Is delta gsh1 synthetic lethal with mutations in genes coding for mitochondrial or cytosolic ISC? We
now show (see below) that GRX3 and GRX4 are synthetic lethal with GSH1.

At best, their slight decrease in GSH concentration required for complementation (new Fig. S7F) could be considered as an indication of synthetic sickness, not synthetic lethality. The authors forgot to describe the new strains in M&M section.

2) The colony size difference between aerobiosis and anaerobiosis is unavoidable, due to the presence of ergosterol...

I am sorry that the authors did not try to improve the figure 5E, as they managed to do for Fig. 6A versus 6B (also aerobic versus anaerobic plates). Many groups have managed to improve the plates by simply adding ergosterol and Tween 80 to the aerobic plates as well.

3) Use of petite is confusing:...

I am sorry if I also wrote my sentence in a "confusing" way; I did not mean that I couldn't follow their rationale for the use of petite mutants. Rather, I do not find useful to display rho- mutants in the main figure, and send the wt background to supplementary (Fig. S7B). In my opinion, it should have to be the opposite (send the respiratory mutants to supplementary material). In fact, 100 microM Fe seems to allow growth better in the wt than in the rho- background (compare Fig. S7B (WT) with Fig. 5B (petite mutants)). If I understand, by using petite mutants the authors

were trying to avoid decreasing the Fe-dependent rescue phenotype due to the Fe-dependent mtDNA damage, and actually the opposite has occurred.

4) We believe that new Fig. 5D is very useful.

5) 2nd line of page 11: Muhlenhoff reference is missing the year.

Additional correspondence (author) 17 March 2011

You will find attached a word copy of the manuscript that has been slightly edited according to referee 1 requests. I have also included the authors' contributions at your request. I am not sending back the figures and supplementary material as they did not change.

