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H2O2 stress-specific regulation of S. pombe MAPK Sty1 by mitochondrial protein phosphatase Ptc4

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1st Editorial Decision 21 April 2011

Thank you very much for submitting your paper on putative mitochondrial functions of Sty1 in conjunction with the Ptc4-phosphatase upon H2O2-stress for consideration to The EMBO Journal editorial office.
I do enclose the scientific assessment from indeed four scientists below. As you will see, all rank the potential interest in your findings as high. However, they all raise concerns if it comes to detailed molecular mechanisms on docking and cleavage as well as kinetics and amount of Sty-1 mitochondrial localization upon stress stimuli. Lastly, at least some evidence for functional importance in this specific compartment would lend significant support for your proposal. As the hypothesis seems rather provocative and novel, it needs sufficient support to convince the currently still critical referees. Given their constructive criticisms however, we decided to offer you the chance to extend the current dataset during a single round of major amendments. We do realize that this entails significant and challenging work and would thus be prepared to offer more than the limited amount of three month upon your request. I do urge you to take the demands from our referees really serious to avoid disappointments much later in the process.

Please do not hesitate to contact me with in case of further questions or indeed possible extension of the revision deadline (preferably via E-mail).

Finally, I do have to formerly remind you that it is EMBO_J policy to allow one round of revisions only and that the final decision on acceptance or rejection depends on the content and strength of the revised version of your study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Di et al presented functional, biochemical and cellular characterization of Ptc4 in relation with Sty1 regulation. Several observations were made based on different methods of analyses. Ptc4 is localized to mitochondria and is subjected to H2O2 selective regulation in its cleavage. Ptc4 selectively regulates H2O2 induced Sty1 activation in both its amplitude and dynamics. Ptc4 also regulates mitochondrial function and cell metabolism.
These observations are very interesting and can have significant implications in intracellular stress signaling. The overall analyses are comprehensive and thorough. However, there are several important issues the paper did not yet address clearly.
1). Sty1 distribution in mitochondria vs. cytosol/nulei was not changed by Ptc4 mutants or expression. Yet, Sty1 activation dynamics was altered (nuclei accumulation) outside of mitochondria by Ptc4 mutant or expression. The main question is what the functional significance of mitochondrial fraction of Sty1 can be in Ptc4 mediated effects under H2O2 stimulation. Clearly, Sty1 is not the only target of Ptc4 in mitochondria, and expressing cytosolic mutant of Ptc4 failed to restore wildtype function in Sty1 regulation. It is possible a cross-talk between mitochondria Ptc4 and cytosolic Sty1, i.e. through oxidative intermediates without direct involvement of mitochondrial fraction of Sty1. That is the effect of Ptc4 on Sty1 can be rather indirect. In fact, the data from Ptc4-truncation mutant provided further evidence to support that. Despite its interaction, cytosolic expression of Ptc4 failed to regulate Sty1 activation profile. Therefore, authors should provide some evidence that Ptc4 mediated regulation of Sty1 is indeed via its direct interaction within mitochondria. The conclusion summary in Figure 6 should reflect that.
2). The effect of Ptc4 on mitochondria respiration and morphology should be explored better. Direct evidence of mitochondrial respiration level and mitochondrial dynamics markers should be characterized in Ptc4 mutants in response to oxidative stress.

Referee #2:

This manuscript describes, for the first time, an important role of the type 2C protein phosphatase Ptc4 in down-regulation of oxidative-stress induced Sty1 activity (Fig 1). It also demonstrates that oxidative stress specifically inhibits cleavage of the N-terminal MTS (mitochondrial targetting sequence) of Ptc4 (Fig 2). Ptc4 as well as a fraction of Sty1 are localized in the mitochondria (Fig 3). These are clearly demonstrated.

They also suggest that the N-terminus of Ptc4 contains a docking site for Sty1. If so, Ptc4 should interact with Sty1 only after oxidative stress. This will neatly explain why Ptc4 is important for Sty1 inactivation only after oxidative stress. However, the data in Figure 4, which presumably prove these ideas, are less than convincing.

Especially, interpretation of the data in Figure 4A needs more attention. They observed that there was more co-IP'ed Sty1 in lane 4 than in lane3, and that the Ptc4 precursor was present in lane 4, but not in lane 3. From these observations, they suggested that the full-length precursor form of Ptc4 might have greater affinity for Sty1 than the cleaved form. The quality of data, however, does not warrant such suggestion, and leaves room for alternative interpretations. Most important, when Input and IP data are compared, it is clear that the efficiencies of IP vary, not only among samples, but also between processed and precursor Ptc4. For example, in lanes 6 and 8, the amount of the IP'ed Ptc4 is much less than that in lanes 5 and 7, even though the amount of Ptc4 is about the same in Input. Also, in lane 4, full-length Ptc4 is significantly underrepresented in IP than in Input. Why are there such variations? Without a clear explanation of these variations, or another experiment without

such variations, these data in Figure 4A cannot be interpreted.

Figure 5 demonstrates that the triple mutation (R8, 10, 27A) in MTS abrogates the ability of Ptc4 to dephosphorylate Sty1, but not the ability to support the growth on non-fermentable carbon source. Thus, it is suggested that Ptc4 has multiple mitochondorial-associated functions. Although these data do not indicate what the other substrates might be, they clearly show that the triple-mutant is fully functional toward certain substrates, but not toward Sty1.

In conclusion, this reviewer feels that this manuscript has one major weakness, namely that the crucial hypothesis that MTS contains a Sty1 docking site, is not yet convincingly demonstrated.

Referee #3:

This manuscript investigates the relationship between the Sty1 MAPK and Ptc4 PP2C phosphatase in fission yeast. It reports that Sty1 activation is prolonged in a ptc4 mutant specifically in response to oxidative stress. Ptc4 localizes in the mitochondria via a N-terminal mitochondrial targeting sequence (MTS) that is cleaved upon import. Cleavage of the MTS is inhibited by oxidative stress. Evidence is provided that a fraction of Sty1 is localized in the nucleus. Additional studies indicate that the MTS promotes Ptc4 binding to Sty1, suggesting a mechanism by which Ptc4 specifically regulates Sty1 in response to oxidative stress.

The conclusions from these studies are novel and provocative, as they indicate that Sty1 functions in the mitochondria and potentially reveal an unexpected mechanism for specifically regulating mitochondrial Sty1 through a multifunctional MTS sequence that also serves as Sty1 docking domain. Much of the data are quite strong, but those concerning the most interesting conclusions are marginal, as discussed below.

One of the most important conclusions from this study is that Sty1 functions in the mitochondria. The idea is intriguing but I find the data to be insufficient to support such a significant finding. A fractionation assay suggests that a subpopulation of Sty1 localizes in the mitochondria (Fig. 3A), but this assay is prone to contamination of fractions (e.g. nuclear proteins appearing in cytoplasmic fraction). There is no evidence for transport of Sty1 into the mitochondria in response to stress, unlike its transport into the nucleus. Nor does the localization of Sty1 in the mitochondria depend on its phosphorylation, from which the authors conclude that there is likely an intact MAPK signaling module in the mitochondria. This is a speculation too far. EM staining of purified mitochondria suggests that Sty1 may localize in the nucleus, although it is strange that the mitochondria look very different from the control sample (Fig. 3F). This does not appear to be an appropriate control. The gold standard would be live cell microscopy of fluorescently tagged proteins with appropriate co-localization controls. Any mitochondrial enrichment Sty1 or other components of the MAPK signaling module should be obvious.

Hopefully at least one of the reviewers is an expert in the mitochondrial localization of proteins. If not, the editors should seek such an expert for their opinion.

Another troubling observation was the absence of any (never mind "strong") increase in the mitochondrial Sty1 phosphorylation in response to oxidative stress (Fig. 3B). The authors suggest that technical limitations of the assay may prevent detection of the phsopho-Sty1. This is insufficient for such an important question of whether Sty1 functions in the mitochondria.

The data establish that Sty1 activation and nuclear localization in response to oxidative stress are strikingly prolonged in a ptc4 mutant. However, from my assessment of the data it appears that neither of these results can be attributed to a defect in dephosphorylation of the mitochondrial fraction of Sty1, which from the data presented must only represent a small fraction of the total Sty1. Indeed, the authors note that their data shows that Ptc4 likely has Sty1 unrelated functions that are critical for responding to oxidative stress. These functions most likely explain the physiological effects of the ptc4 mutation. At least there are no convincing data to the contrary.

The R8A, R10A, R27A mutant is potentially informative on this question. It grows in non-

fermentable carbon sources, suggesting that it retains significant function. Sty1 phosphorylation is prolonged in this mutant, but nuclear localization is not prolonged. The authors propose that this can be explained by a prolonged activation of Sty1 in the mitochondria. This speculation needs to be tested. Measuring the phosphorylation status of the mitochondrial and non-mitochondrial pools of Sty1 in this mutant would test this hypothesis. It will also be important to test whether this mutant is sensitive to oxidative stress or has any other phenotypes similar to the ptc4 null.

Another important piece of data is that showing that Ptc4 can be co-precipitated with Sty1 (Figure 4A) and this largely depends on the formation of unprocessed Ptc4 in response to oxidative stress. But as the authors note, the unprocessed Ptc4 is precipitated inefficiently for unknown reasons, which makes one wonder whether the unprocessed Ptc4 that is poorly precipitated can explain the increased Sty1 signal in lane 4. The Sty1 signals are so weak in this assay, and with the non-linearity of western blot signals, it really would make me nervous about these results. A stronger Sty1 signal with quantitation and standard error seems to be necessary.

Referee #4:

The authors provide an interesting observation that links the Sty1 MAPK with the mitochondria and its regulation by the protein phosphatase Ptc4 in oxidative stress but not other type of stresses. In addition, they provide evidence of the regulation of Ptc4 processing that could be important for its affinity and regulation of the MAPK.
However, the article fails short in providing the molecular basis of Ptc4 regulation by cleavage and its relationship to oxidative stress. For instance, it is not defined who and how Ptc4 is cleaved and whether it depends on Sty1. The association of Ptc4 and Sty1 in figures 4 and 5 is not always consistent and clear.

I also found not sufficiently convincing the fact that there are many aspects of the manuscript that are not coherent with the hypothesis presented here. I do not understand how Ptc4 can control the down-regulation of Sty1 in response to oxidative stress at similar kinetics than in response to other stresses when only a minor fraction of Sty1 is present at the mitochondria and that Ptc4 is restricted there. It is not clear why there is strong nuclear accumulation of Sty1 at the nucleus upon oxidative stress but its down-regulation should occur at the mitochondria based on their model.

Therefore, I feel that at least at this stage the article is not suitable for EMBOJ.

I believe that suitability could change if the authors could provide additional and solid evidence clarifying the regulation of Ptc4 cleavage and its physiological role together with a good experimental support that defines the molecular mechanism by which Sty1 can be down-regulated at the mitochondria in oxidative stress but display similar down-regulation in response to other stresses.

1st Revision - Authors' Response 07 October 2011

We thank you for your time and effort in considering our manuscript. We would also like to thank the reviewers for their time and their constructive comments. We believe that addressing these has considerably strengthened our manuscript.

Our new data can be summarised as follows:

1. IPs showing a robust interaction between Sty1 and Ptc4 that is dramatically increased upon oxidative stress and abrogated by mutating the MTS of Ptc4.
2. Hyper-activation of mitochondrial Sty1 in both the *ptc4* deletion and the triple arginine *ptc4* mutants.
3. Quantitative oxygen consumption data showing that respiration is severely compromised in the *ptc4* deletion and partially compromised in the triple mutant.

4. Compromised respiration in a *sty1* deletion mutant.
5. Ptc4 cleavage is not dependent upon Sty1.
6. Ptc4 cleavage is dependent upon MPP.
7. Ptc4 can also affect the dynamics of mitochondrial morphology changes upon stress.

We have listed our responses to the individual points raised by the reviewers below; however to avoid repetition, we start with two general responses to all the reviewers firstly addressing the IP data and secondly discussing a possible indirect mechanism by which loss of Ptc4 affects Sty1, as all the reviewers had commented on these issues.

General Response (A) to all reviewers regarding the data in Figure 4A (IP between Sty1 and Ptc4)

We have spent a great deal of time optimising the immuno-precipitations between Ptc4 and Sty1 and now show a more robust interaction (Figure 4A). Moreover, we now routinely see similar amounts of Ptc4 immuno-precipitated. The new data is representative of three independent IPs and we see a clear difference between the amount of Sty1 co-IPed in the absence and presence of stress. We emphasise that our study is the first to show an in vivo interaction between Sty1 and a phosphatase, where both proteins are wild type in sequence and expressed at endogenous levels. Previous studies have identified interactions between Sty1 and its regulatory phosphatases but these have resulted from the over-expression of one or both of Sty1 and its phosphatases, or in some cases through the use of a phosphatase-dead allele to trap the interaction (Genes and Dev, 13:1653-63; Nature 378; 739-743). The increased interaction that we see is not due to a stress-induced modification of Ptc4 or Sty1 since the greater affinity between full length Ptc4 and Sty1 is also observed in vitro where the proteins have not been subject to any stress-dependent modification. Moreover, when mutations are made in this N-terminal region, the interaction, both in vivo and in vitro, is abrogated.

The main focus of this work is to show that a pool of Sty1 exists in the mitochondria and that it is directly regulated by a mitochondrial phosphatase. We show three lines of evidence supporting this conclusion: (1) A direct interaction between Sty1 and Ptc4 as demonstrated by a co-IP; (2) Sty1 and Ptc4 associate with the same mitochondrial sub-fraction; (3) H_2O_2 regulates Ptc4 specifically and this promotes the retention of the MTS which preferably associates with Sty1 (demonstrated by both in vivo and in vitro analysis).

General Response (B) to all reviewers regarding a possible indirect effect of loss of Ptc4 upon Sty1 activation

We agree with the reviewers that Sty1 might be affected in more than one way by the loss of Ptc4. We suggest that Ptc4 directly down-regulates Sty1 in the mitochondria (Figure 4A) and that cytoplasmic Sty1 might be indirectly affected by loss of mitochondrial Ptc4 thus explaining why we observe prolonged localization of Sty1 in the nucleus in a ptc4 deletion mutant. We have previously mentioned this in the discussion and have retained this explanation. We have also modified Figure 6 (now Figure 7) by adding a new model (shown in Figure 7C) as suggested by Reviewer 1 to reflect the indirect mechanism by which Ptc4 may also be affecting Sty1. We have also altered the title of the section describing the data in Figure 6 as follows: “Loss of Ptc4 may affect Sty1 directly through loss of dephosphorylation and also indirectly due to disruption of other mitochondrial functions of Ptc4”.

In support of our model is data from the triple arginine mutant of Ptc4, *(ptc4R8,10,27A)*. In this mutant, Sty1 is no longer localized to the nucleus to the same extent as that observed in the deletion mutant but we still see hyper-activation of Sty1. We had proposed in our original submission that this might be due to the mitochondrial fraction of Sty1 not being down-regulated by this mutant version of Ptc4. We now provide evidence to support this hypothesis. In our new data shown in Figure 5E, we show that indeed, Sty1 remains hyper-activated in the mitochondria of the triple arginine mutant consistent with our hypothesis.

Our new quantitative data demonstrating that loss of Ptc4 gives rise to defects in oxidative phosphorylation strengthens our claim that this phosphatase has other mitochondrial targets. We appreciate that understanding the mechanism of this indirect effect will be important moving forward with this project; however this will require the identification of Ptc4’s other mitochondrial

targets along with a detailed analysis of how loss of such regulation affects mitochondrial functions and thus feel that it is outwith the scope of this current work.

Referee #1:

These observations are very interesting and can have significant implications in intracellular stress signaling. The overall analyses are comprehensive and thorough. However, there are several important issues the paper did not yet address clearly.

1). Sty1 distribution in mitochondria vs. cytosol/nulei was not changed by Ptc4 mutants or expression. Yet, Sty1 activation dynamics was altered (nuclei accumulation) outside of mitochondria by Ptc4 mutant or expression. The main question is what the functional significance of mitochondrial fraction of Sty1 can be in Ptc4 mediated effects under H2O2 stimulation. Clearly, Sty1 is not the only target of Ptc4 in mitochondria, and expressing cytosolic mutant of Ptc4 failed to restore wildtype function in Sty1 regulation. It is possible a cross-talk between mitochondria Ptc4 and cytosolic Sty1, i.e. through oxidative intermediates without direct involvement of mitochondrial fraction of Sty1. That is the effect of Ptc4 on Sty1 can be rather indirect. In fact, the data from Ptc4-truncation mutant provided further evidence to support that. Despite its interaction, cytosolic expression of Ptc4 failed to regulate Sty1 activation profile. Therefore, authors should provide some

evidence that Ptc4 mediated regulation of Sty1 is indeed via its direct interaction within mitochondria. The conclusion summary in Figure 6 should reflect that.

Please see the General Response (B) to all reviewers regarding a possible indirect effect of loss of Ptc4 upon Sty1 activation.

With our improved IPs, we found that the ptc4 truncation mutant (ND1-28Ptc4), which is localized in the cytoplasm, could still bind to low levels of Sty1 in the absence but not presence of stress. This binding is always much less that that observed between wild type Ptc4 (which is mitochondrial) and Sty1. We did not discuss this IP in the original submission but merely included it in the figure as we had shown other data relating to this mutant elsewhere in the manuscript. We feel that the IP data is hard to interpret as this form of Ptc4 is artificially localized to the cytoplasm and therefore we have removed it. The weak interaction between Sty1 and ND1-28Ptc4, in the absence of stress, is probably a result of the fact that this cytoplasmic version of Ptc4 now has a bigger pool of Sty1 to interact with. As we show in our in vitro assay (in Figure 4B), Ptc4 lacking the MTS has a much weaker affinity for Sty1. Our data suggest that Sty1 is hyperactivated in this mutant due to a lack of down-regulation of mitochondrial Sty1 coupled with the mitochondrial defects caused by loss of Ptc4 in this organelle promoting activation of cytoplasmic Sty1 in a similar manner to the ptc4 deletion mutant.

2). The effect of Ptc4 on mitochondria respiration and morphology should be explored better. Direct evidence of mitochondrial respiration level and mitochondrial dynamics markers should be characterized in Ptc4 mutants in response to oxidative stress.

In this revised manuscript, we have examined mitochondrial respiration directly by measuring oxygen consumption. This clearly shows that Ptc4 is essential for respiration. We have also performed these experiments in the *ptc4R8,10,27A* mutant which is still competent to respire, albeit at a reduced rate. These experiments were attempted under conditions of oxidative stress but the oxygen released from the decomposition of H_2O_2 meant that it was not possible to interpret the data (this will be a combination of spontaneous decomposition of H_2O_2 as well as detoxification by catalase).

Regarding the question of mitochondrial morphology, we have examined this by tagging Cox4 with GFP in a *ptc4D* mutant in the presence and absence of oxidative stress. These data are now shown in

Supplementary Figure 3. Upon oxidative stress in wild type, the mitochondria appear to form aggregates (Figure 2E). This still occurs in a *ptc4D* mutant but with slower kinetics. We do not know the reason for this but suspect that Ptc4 has a number of different mitochondrial targets. We feel that determining the biology underpinning these morphological events, while interesting, is outside the scope of this current study.

Referee #2:

This manuscript describes, for the first time, an important role of the type 2C protein phosphatase Ptc4 in down-regulation of oxidative-stress induced Sty1 activity (Fig 1). It also demonstrates that oxidative stress specifically inhibits cleavage of the N-terminal MTS (mitochondrial targeting sequence) of Ptc4 (Fig 2). Ptc4 as well as a fraction of Sty1 are localized in the mitochondria (Fig 3). These are clearly demonstrated.

They also suggest that the N-terminus of Ptc4 contains a docking site for Sty1. If so, Ptc4 should interact with Sty1 only after oxidative stress. This will neatly explain why Ptc4 is important for Sty1 inactivation only after oxidative stress. However, the data in Figure 4, which presumably prove these ideas, are less than convincing.

Especially, interpretation of the data in Figure 4A needs more attention. They observed that there was more co-IP'ed Sty1 in lane 4 than in lane3, and that the Ptc4 precursor was present in lane 4, but not in lane 3. From these observations, they suggested that the full-length precursor form of Ptc4 might have greater affinity for Sty1 than the cleaved form. The quality of data, however, does not warrant such suggestion, and leaves room for alternative interpretations. Most important, when Input and IP data are compared, it is clear that the efficiencies of IP vary, not only among samples, but also between processed and precursor Ptc4. For example, in lanes 6 and 8, the amount of the IP'ed Ptc4 is much less than that in lanes 5 and 7, even though the amount of Ptc4 is about the same in Input. Also, in lane 4, full-length Ptc4 is significantly underrepresented in IP than in Input. Why are there such variations? Without a clear explanation of these variations, or another

experiment without such variations, these data in Figure 4A cannot be interpreted.

Please see the General Response (A) to all reviewers regarding the improved IPs between Sty1 and Ptc4.

Regarding the underrepresentation of full length Ptc4 in the IP versus the input, this is something we consistently see. Below we show another IP to illustrate this point. Here we have also immunoprecipitated Ptc4 using extracts from a later time point of stress to see if we might recover more full length protein as there is more of it in the input at this point. However, we consistently recover less full length protein from these samples. We do not understand the reason for this but suggest it may be related to the mitochondrial morphological changes that we observe upon stress. It could be that different complexes are formed, or conformational changes occur in Ptc4 that preclude access to the epitope tag. This is now mentioned in the text.

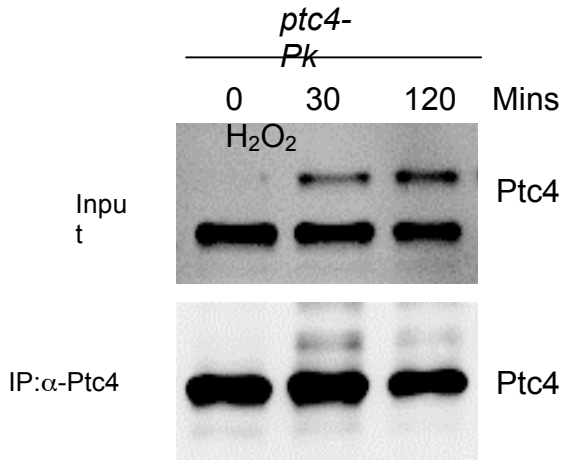


Figure 5 demonstrates that the triple mutation (R8, 10, 27A) in MTS abrogates the ability of Ptc4 to dephosphorylate Sty1, but not the ability to support the growth on non-fermentable carbon source. Thus, it is suggested that Ptc4 has multiple mitochondrial-associated functions. Although these data do not indicate what the other substrates might be, they clearly show that the triple-mutant is fully functional toward certain substrates, but not toward Sty1.

In conclusion, this reviewer feels that this manuscript has one major weakness, namely that the crucial hypothesis that MTS contains a Sty1 docking site, is not yet convincingly demonstrated.

We have clearly shown that Ptc4 regulates respiration (new data in Figure 6C) and agree with the reviewer that other substrates of Ptc4 exist, and that it will be important, in the future, to identify the targets relevant for this phenotype. The current study, however, centres on Ptc4's ability to regulate a mitochondrial fraction of Sty1 and we now provide robust in vivo and in vitro evidence that the MTS of Ptc4 is responsible for promoting this regulation (detailed in General Response A).

Referee #3:

This manuscript investigates the relationship between the Sty1 MAPK and Ptc4 PP2C phosphatase in fission yeast. It reports that Sty1 activation is prolonged in a ptc4 mutant specifically in response to oxidative stress. Ptc4 localizes in the mitochondria via a N-terminal mitochondrial targeting sequence (MTS) that is cleaved upon import. Cleavage of the MTS is inhibited by oxidative stress. Evidence is provided that a fraction of Sty1 is localized in the nucleus. Additional studies indicate that the MTS promotes Ptc4 binding to Sty1, suggesting a mechanism by which Ptc4 specifically regulates Sty1 in response to oxidative stress.

The conclusions from these studies are novel and provocative, as they indicate that Sty1 functions in the mitochondria and potentially reveal an unexpected mechanism for specifically regulating mitochondrial Sty1 through a multifunctional MTS sequence that also serves as Sty1 docking domain. Much of the data are quite strong, but those concerning the most interesting conclusions are marginal, as discussed below.

One of the most important conclusions from this study is that Sty1 functions in the mitochondria. The idea is intriguing but I find the data to be insufficient to support such a significant finding. A fractionation assay suggests that a subpopulation of Sty1 localizes in the mitochondria (Fig. 3A), but this assay is prone to contamination of fractions (e.g. nuclear proteins appearing in cytoplasmic fraction).

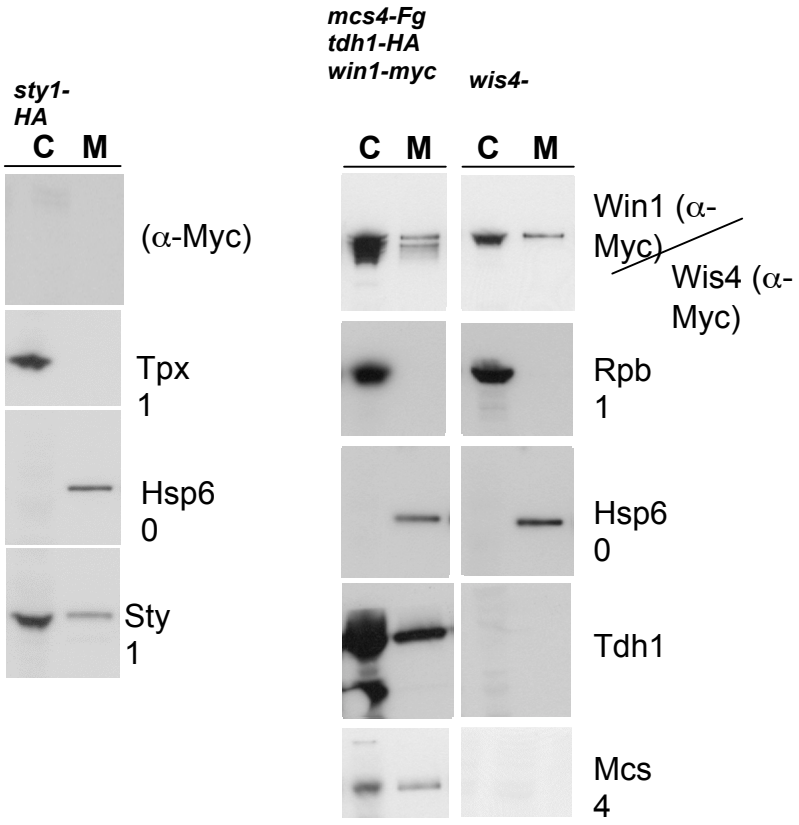
We used Rpb1 as a control because this protein has been shown to be a component of both the cytoplasm and the nucleus (http://www.riken.jp/SPD/41/41B01.html). Therefore, lack of Rpb1 in our mitochondrial extracts demonstrates that they are relatively free of both nuclear and cytoplasmic contamination. We have included other purifications in Supplementary Figure 7 which clearly show both Tpx1 and Rpb1 do not contaminate the mitochondrial fraction as well as using antibodies

against Mts4, a proteasome component which is also known to be both cytoplasmic and nuclear (EMBO J. 1998 Nov 16;17(22):6465-76); we chose Mts4 and Tpx1 as controls because both proteins are highly abundant.

We do appreciate that such a novel finding warrants thorough investigation which is why we chose to verify our findings with immuno-electron microscopy. We believe that the specific Sty1 signal that we observe in slices through fixed mitochondria, strongly and directly supports our conclusion that Sty1 can be located in the mitochondria and that our biochemical observations are not due to contamination by other cellular components.

There is no evidence for transport of Sty1 into the mitochondria in response to stress, unlike its transport into the nucleus. Nor does the localization of Sty1 in the mitochondria depend on its phosphorylation, from which the authors conclude that there is likely an intact MAPK signaling module in the mitochondria. This is a speculation too far.

We have observed other components of the Sty1 MAPK signalling cascade in our fractionated mitochondria. Some of this data is shown below including the MAPKKKs Wis4 and Win1, Tdh1 and Mcs4. We have not yet corroborated this with any immuno-EM as it takes time to get the right antibodies/tag/fixation conditions; however, we feel that our existing data is sufficient to allow us to speculate about their partial localization to mitochondria.



C – cytoplasmic fraction; M –mitochondrial fraction. The fractions represent extracts from the same number of cells. Components of the MAPK cascade were detected by immuno-blotting. This is representative of three independent experiments.

EM staining of purified mitochondria suggests that Sty1 may localize in the nucleus, although it is strange that the mitochondria look very different from the control sample (Fig. 3F).

We have now included mitochondria from wild type cells that are of a similar appearance. How the mitochondria appear depends firstly on their orientation with respect to the plane of sectioning, so some can look elongated whereas others look round; and secondly, their size can vary quite widely depending on the balance between mitochondrial fission and fusion.

This does not appear to be an appropriate control. The gold standard would be live cell microscopy of fluorescently tagged proteins with appropriate co-localization controls. Any mitochondrial enrichment Sty1 or other components of the MAPK signaling module should be obvious.

We made several attempts with such a microscopic approach but after consultation with experts in our imaging facility we decided against pursuing this type of analysis due to contrast problems. The amount of endogenous Sty1 in the cytoplasm prevented us achieving a suitable image via wide-field restorative microscopy or spinning disk confocal microscopy. We attempted co-localisation analysis after image deconvolution but as endogenous Sty1 is a real event rather than a background issue due to processing of the samples, image deconvolution kept the background cytoplasmic detail along with the mitochondrial detail. Images were captured with a range of magnifications to improve optical sectioning ability under confocal microscopy and even when using a x100 objective (1.45NA) the endogenous background was still an issue. Using the Deltavision and the x100 1.45 NA objective lens, the size per pixel is 0.15 microns per pixel so for two objects to be separate they must be at least 0.3 microns apart. If two proteins are less than 0.15 microns apart, (entirely possible for a cytoplasmic protein and a protein associated with the inner mitochondrial membrane), they might not co-localise in real life but may appear to do so as this resolution is not molecular resolution. As we were mindful of over-processing the data to achieve a result, we thought it suitable to seek other methods of characterisation. As our immuno-EM data represents slices through purified mitochondria, we considered this to be a more robust method than immuno-fluorescence microscopy.

Another troubling observation was the absence of any (never mind "strong") increase in the mitochondrial Sty1 phosphorylation in response to oxidative stress (Fig. 3B). The authors suggest that technical limitations of the assay may prevent detection of the phsopho-Sty1. This is insufficient for such an important question of whether Sty1 functions in the mitochondria.

As stated in the previous version (and retained in this one on p9), it is very hard to observe Sty1 activation in wild type mitochondria as the methodology for preparing the mitochondria involves lengthy processing to remove the cell wall followed by harvesting and washing of the cells before they are placed on ice to stop any further metabolism. This is enough time for the cells to adapt to the stress and for Sty1 activation to be down-regulated. Moreover, the procedures used to prepare the cells for the zymolyase treatment (harvesting and washing), and the treatment itself, lead to weak activation of Sty1 so that even with cells grown under basal conditions there is weak activation of Sty1 which may mask further activation upon stress. The exception to this is in purified mitochondria from the ptc4 deletion and triple mutants where we have essentially trapped Sty1 in its activated state (Figures 3B and 5E). In more recent experiments, we have trialled different lysis buffers containing various combinations of phosphatase inhibitors and can sometimes observe weak activation in wild type mitochondria but the result is not consistent and so we have not included this data.

Regarding whether Sty1 functions in the mitochondria, we appreciate that it will be important to identify targets of the kinase in this organelle though this is not a trivial undertaking. However, we now show that Sty1 clearly regulates oxidative phosphorylation (Figure 6C). While we cannot say for sure if this is a direct effect, it is consistent with our hypothesis that Sty1 regulates mitochondrial proteins directly. Our previous genomic profiling data (Chen et al., 2003, Mol Biol Cell.14(1):214-29) did not identify any known ETC components as being under the transcriptional control of Sty1.

The data establish that Sty1 activation and nuclear localization in response to oxidative stress are strikingly prolonged in a ptc4 mutant. However, from my assessment of the data it appears that neither of these results can be attributed to a defect in dephosphorylation of the mitochondrial fraction of Sty1, which from the data presented must only represent a small fraction of the total Sty1. Indeed, the authors note that their data shows that Ptc4 likely has Sty1 unrelated functions that are critical for responding to oxidative stress. These functions most likely explain the physiological effects of the ptc4 mutation. At least there are no convincing data to the contrary.

The R8A, R10A, R27A mutant is potentially informative on this question. It grows in non-fermentable carbon sources, suggesting that it retains significant function. Sty1 phosphorylation is prolonged in this mutant, but nuclear localization is not prolonged. The authors propose that this can be explained by a prolonged activation of Sty1 in the mitochondria. This speculation needs to be

tested. Measuring the phosphorylation status of the mitochondrial and non-mitochondrial pools of Sty1 in this mutant would test this hypothesis. It will also be important to test whether this mutant is sensitive to oxidative stress or has any other phenotypes similar to the ptc4 null.

We have now tested the mitochondrial pools of Sty1 in the ptc4 deletion and the triple arginine mutants. In both cases, Sty1 is activated to a greater extent than wild type (Figures 3B and 5E). It is not possible to fractionate nuclei in these experiments as they co-pellet with the unbroken cells. This is why we examined nuclear activation using immuno-fluorescence microscopy as it is known from previous work that Sty1 becomes nuclear when activated (Genes Dev. 1998, 12(10):1464-73). We clearly see that Sty1 does not remain in the nucleus in the triple mutant to the same extent as it does in the deletion mutant (Figure 6D) thus suggesting that the proposed indirect effect is not as strong in this mutant as it is in the delete. As detailed in our General Response to Reviewers (B), we have included a figure and text in the discussion to explain how this prolonged nuclear accumulation is most likely an indirect effect of Ptc4 loss. Our new respirometry data is also consistent with this idea as oxidative phosphorylation is compromised to a far greater extent in the ptc4 deletion mutant than in the triple mutant (Figure 6C).

We have carried out the oxidative stress sensitivity experiment. Even though the triple mutant can still respire, it is sensitive to this stress as is the deletion mutant. H_2O_2 sensitivity is a phenotype known to be associated with mitochondrial defects. This new data is shown in Figure 6A.

Another important piece of data is that showing that Ptc4 can be co-precipitated with Sty1 (Figure 4A) and this largely depends on the formation of unprocessed Ptc4 in response to oxidative stress. But as the authors note, the unprocessed Ptc4 is precipitated inefficiently for unknown reasons, which makes one wonder whether the unprocessed Ptc4 that is poorly precipitated can explain the increased Sty1 signal in lane 4. The Sty1 signals are so weak in this assay, and with the non-linearity of western blot signals, it really would make me nervous about these results. A stronger Sty1 signal with quantitation and standard error seems to be necessary.

Please see General Response to reviewers (A).

Referee #4:

The authors provide an interesting observation that links the Sty1 MAPK with the mitochondria and its regulation by the protein phosphatase Ptc4 in oxidative stress but not other type of stresses. In addition, they provide evidence of the regulation of Ptc4 processing that could be important for its affinity and regulation of the MAPK.

However, the article fails short in providing the molecular basis of Ptc4 regulation by cleavage and its relationship to oxidative stress. For instance, it is not defined who and how Ptc4 is cleaved and whether it depends on Sty1.

We now show that the cleavage of Ptc4 is dependent upon the major mitochondrial matrix protease MPP which plays a well defined role cleaving amino-terminal mitochondrial targeting sequences in a number of organisms. This was shown by using a mas1 mutant which fails to cleave Ptc4 in an in vitro import assay. This new data is shown in Supplementary Figure 9.

Taken together with the fact that Ptc4 has an MTS consistent with other targets of MPP, we conclude that MPP is likely to be the protease responsible for the cleavage of this phosphatase. Consistent with what is known about MPP in other organisms, our data demonstrate that Ptc4 is cleaved inside the mitochondria as uncleaved Ptc4 in the *mas1* experiment is still protected from protease K digestion suggesting that import is competent but cleavage is blocked.

We have tried to determine how oxidative stress might affect MPP but this is not trivial and our experiments to date have ruled out oxidation of Mas2 cysteine residues as a possible mechanism (Mas1 does not possess any cysteines). Whilst the mechanism is an important detail, we feel that it is a matter for future studies.

We have now clearly demonstrated that the cleavage of Ptc4 is not dependent upon Sty1. This new data is shown in Supplementary Figure 1.

The association of Ptc4 and Sty1 in figures 4 and 5 is not always consistent and clear.

Please see the General Response (A) to all reviewers regarding the improved IP between Sty1 and Ptc4.

I also found not sufficiently convincing the fact that there are many aspects of the manuscript that are not coherent with the hypothesis presented here. I do not understand how Ptc4 can control the down-regulation of Sty1 in response to oxidative stress at similar kinetics than in response to other stresses when only a minor fraction of Sty1 is present at the mitochondria and that Ptc4 is restricted there. It is not clear why there is strong nuclear accumulation of Sty1 at the nucleus upon oxidative stress but its down-regulation should occur at the mitochondria based on their model.

Please see the General Response (B) to all reviewers regarding a possible indirect effect of loss of Ptc4 upon Sty1 activation.

I believe that suitability could change if the authors could provide additional and solid evidence clarifying the regulation of Ptc4 cleavage and its physiological role together with a good experimental support that defines the molecular mechanism by which Sty1 can be down-regulated at the mitochondria in oxidative stress but display similar down-regulation in response to other stresses.

It is possible that upon other stresses, mitochondrial Sty1 is not activated particularly strongly. Alternatively, other phosphatases may be present that can down-regulate Sty1 under such conditions. Indeed, potential MTS regions can be identified in both Azr1 and Pyp1 which are types of phosphatases that are known to act upon Sty1 (with Pyp1 already known to act upon cytoplasmic Sty1). It is clear from what is known about MAP kinases in a range of systems, that their activation and regulation is dependent upon the precise context and activating conditions. It is therefore necessary to determine the specific regulators and patterns of regulation in each specific circumstance. Here we have found a specific phosphatase that physically interacts with Sty1 in a stress-specific manner and clearly regulates its activity under those conditions.

Pre-Acceptance Letter 28 October 2011

I am happy to inform you that having received necessary external input on the issue of mitochondrial localization of Sty1, your paper will be accepted for publication in The EMBO Journal. The editorial office will soon be in touch with the official paperwork.

As we do encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We thus offer the possibility to publish a single PDF file comprising the original, uncropped and unprocessed scans at least for the key data of your paper. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. This PDF will be published online with the article as a supplementary "Source Data" file. Please let me know if you have any questions about this policy or check this link for a recent example (http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html).

Please allow me to congratulate to your paper on this occasion and I am very much looking forward to your response whether or not you are going to support our source data initiative.

Yours sincerely,

Editor
The EMBO Journal

Referee reports:

Report of Referee 3 (received 24 October 2011)

My previous comments concerned 2 major issues:

1. Does Sty1 really localize in the mitochondria?
2. Are the effects of Ptc4 on Sty1 direct or indirect?

The authors are confident that their mitochondrial fractions are pure, and so they have no doubt that Sty1 localizes in the mitochondria. I am more skeptical of subcellular fractionation and was hoping to see an independent confirmation of their findings. They contend that the EM studies satisfy this requirement, but as they presumably know, these experiments were done with the same subcellular fractionation protocol, and thus are not really an independent experiment.

The authors accept that some of the effects of Ptc4 on Sty1 may be indirect. I think it is obvious that much of the effects must be indirect, since the total pool of Sty1 is strongly affected by Ptc4 deficiency. The contention that Ptc4 directly regulates Sty1 relies on the co-immunoprecipitation experiment. The interaction is weak, as it should be (see next paragraph), and in vitro it does not strictly depend on the MTS sequence that is supposed to mediate binding in vivo. It is a small peg on which to hang a large hat.

Several of the reviewers were unconvinced by the Ptc4-Sty1 co-immunoprecipitation data. New data in Figure 4A strengthen the evidence. However, describing the interaction as more robust seems a bit much. I think it would be fair to say that typical phosphatase-substrate interactions are never robust. The authors imply that their data are better because they did not rely on a phosphatase-dead allele to trap the interaction. This comment misses the point of the experiment. Trapping the interaction strengthens the evidence because it shows that the interaction is of a phosphatase binding its substrate.

In summary, the manuscript provides persuasive evidence that Ptc4 is a mitochondrial protein that is required for the oxidative stress response and therefore can indirectly affect Sty1 phosphorylation. Subcellular fractionation data suggest that some Sty1 localizes in the mitochondria, although it is not enriched relative to the cytoplasm. Ptc4 may associate with Sty1, but it is unclear whether Ptc4 dephosphorylates Sty1 in vivo.

Editor asked for further external input 26 October 2011

I would like to get some input from you on a revised paper that is hard to reach a final decision on. It concerns the Sty1/Ptc4 MAP-kinase/phosphatase pair that our authors propose to function in the mitochondria (you were kind enough to advice on in the first place).

I reviewed the paper and in fact four scientists were rather supportive, pending major revisions. In particular, the evidence for Sty1's mitochondrial localization and its interaction with Ptc4 seems of major concern. The authors tried to overcome these issues experimentally (please refer to their attempts in their general and specific response to ref#3).
Still, I would prefer the input of a mitochondrial expert, specifically on the crucial figure 3 (biochemical fractionation/various treatments to determine Sty1's mitochondrial localization and EM of mitochondria from Sty1-myc expressing cells) before reaching a final verdict.

External advice received 28 October 2011

I carefully reviewed the data presented in the paper, as well as the discussion of the authors in their response to reviewers' comments, concerning the localization of Sty1 to mitochondria.

The presented data strongly suggest that this protein is integral in the inner membrane of the organelle (see the carbonate extraction and the immunoreactivity in the cristae found in the immuno-EM). One reviewer is concerned that this last piece of evidence is weakened by a potential nuclear contamination. However, the protein is not on the surface of the organelle, but associated with the cristae membrane, suggesting that import and insertion had occurred. Therefore, if the immunodecoration was an artifact of the nuclear contamination, one would have to assume that the protein had to be anyway imported into mitochondria, post release from the nuclear fraction.

I believe that the sucrose gradient purification of mitochondria (devoid of cytosolic contaminants) and the immunoEM are acceptable evidence for a mitochondrial localization. My advice is therefore to proceed with the paper and to publish it.