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Stress-induced OMA1 activation and autocatalytic turnover regulates OPA1-dependent mitochondrial dynamics

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Editor: Andrea Leibfried

1st Editorial Decision

03 September 2013

Thank you for submitting your manuscript to The EMBO Journal. Dr. Andrea Leibfried is the handling editor on this submission, but as she is away on vacation, I have stepped in to avoid delays. Your manuscript has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and insightful. While referee 1 raises relative minor concerns, both referees #2 and 3 raise more significant ones. They both find that some of the conclusions are too preliminary and need further support. Should you be able to address the concerns in full, then I would like to invite you to submit a revised version of the manuscript. As you know it is EMBO Journal policy to allow only a single round of revision and it is therefore important to address the raised concerns at this stage.

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

REFeree COMMENTS

Referee #1

In this manuscript Baker et al have analyzed the role of the OMA1 protease in the processing of the GTPase OPA1. They find that stress-induced activation of OMA1 is critical for this process. The activation of OMA1 by cellular stress and the identification of a stress-sensing region within the

OMA1 protein are key observations of the paper. The author's finding on an autocatalytic degradation, significantly extends our knowledge on OMA1 regulation and how it links to mitochondrial dynamics through OPA1. The article is very well written and a pleasure to read. The presented data is of very high quality and the experiments are well controlled. The manuscript by Baker and colleagues presents an impressive analysis on OMA1 function, which provides an unexpected new regulatory principle of mitochondrial dynamics. The article is appropriate for publication in EMBO journal with only minor revisions.

Specific points

1.) In order to show the functionality of Myc-tagged OMA1 (Fig. S1) the authors expressed OMA1-myc in OMA1^{-/-} cells. However, the processing of OPA1 is much less obvious than in previous experiments. Since the loss of L-OPA1 in an OMA1-dependent manner is more obvious after mitochondrial depolarisation, the authors could perform the experiment similar to what they presented in Fig. 1A.

2.) Fig. 7b presents an important experiment. This experiment indicates that OMA1 activity can be obtained expressing simultaneously OMA1E3240-myc and OMA1Δ144-163-myc. However, this assay provides indirect evidence for an interaction. It would support the presented data, if the authors could perform a co-IP experiment of the proteins or show a similar migration in density gradients as presented in Fig. 7A. However, the referee is aware of the fact that that transient or weakly associated complexes might not be stable enough to survive such analyses.

Minor points:

A.) The establishment of OMA1^{-/-} cells using Zinc Finger nucleases is described only briefly in the method section, this should be extended.

B.) Western blots should be labelled with OMA1-myc and not only with Myc.

C.) Fig. 1: It would be helpful to add a graphical image for the different L- and S-OPA1 forms. In the result section the authors state, that stress conditions activate OPA1 causing beside L-OPA1 degradation also fragmentation of the mitochondrial network. Adding a reference or a microscopy image would support this statement.

D.) Fig. 2A: A reduction of OMA1-myc as well as Smac is visible in non-swollen mitochondria upon protease treatment, this should be briefly discussed.

In the methods section, it should be added how much PK was used in the experiment of Fig. 2A.

E.) Fig. 3E: According to the text, detection of OMA1 was neither possible in MEFs nor in HEK293 cells. However, in Fig. 3E and 3F OMA1 and OMA1-myc was decorated; please clarify.

F.) Fig. 3F: Since the induction of OMA1-myc is important for the experimental setup, the time of induction used in Fig. 3E should be stated in the text

Referee #2

This study focuses on the mechanisms by which the zinc metalloprotease Oma1 becomes activated upon mitochondrial dysfunction. As a resident of the inner mitochondrial membrane, the authors had previously reported that the addition of CCCP somehow led to the activation of Oma1, which cleaves the fusion GTPase Opa1 thereby inactivating mitochondrial fusion. However, it was unknown how Oma1 was maintained in an inactive state, and how it would detect the loss in potential to become active. Here the authors continue their investigation into this process and present a series of new findings. The authors now show that Oma1 can become activated by a variety of mitochondrial stressors. They better define the topology and N-terminal import cleavage site of Oma1, confirming that the catalytic domain resides in the IMS rather than the matrix. They show that Oma1 is autocatalytically cleaved and degraded upon the addition of CCCP, presumably providing an opportunity for new Opa1 to become imported should the mitochondria be repaired. The CCCP-induced autocatalytic step is unique to higher organisms, due to an expanded amino- and carboxy-terminal domains. They show that an amino-terminal domain contains positively charged residues that could act as the sensor for the electrochemical potential. The C-terminal expansion appears to be inhibitory since its removal led to the constitutive activation of Oma1. Finally, the

autocatalytic activity of Oma1 is mediated through trans interactions within a larger oligomeric complex. This complex is unchanged upon loss of potential, indicating that the regulation of its activity does not involve overall complex dynamics. The data presented are of very high quality, but some conclusions are preliminary and require further clarification.

1. The authors screen a series of toxins to examine Oma1-dependent cleavage of Opa1. It is difficult to really know what the conclusion here is since the authors did not confirm whether or not the electrochemical potential was lost in each instance of Oma1 activation. Or is ROS generation the critical factor? To test this they would need to add scavengers to confirm the requirement for ROS, and directly quantify the loss in potential in each case. For example, with antimycin A and oligomycin, wouldn't oligomycin hyperpolarize, and the ROS may increase at complex III? Or is it true that together the mitochondria lose potential? The peroxide and paraquat treatments may have led to a loss of potential, or not - it would have to be directly tested. And where does heat shock fit in this paradigm? This experiment seems incomplete and difficult to interpret. The authors rather simply conclude that lots of different stress leads to Oma1 activation. However, with the focus later on the N-terminal positively charged region, it would suggest that electrochemical potential loss is key. I think it is important to better define the event that triggers Oma1 activation.

2. The mutation of 6 lysine/arginines to glutamate in the N-terminal domain blocks the activation of Oma1 cleavage. The authors must test whether the Oma1-6 mutant is properly assembled within the inner membrane. I agree that there is some cleavage of Opa1, suggesting that the protein is properly imported, however it would also be interesting to see whether this construct also migrates in the same oligomeric complex.

3. What is the nature of the Oma1 oligomer? Is there a mitochondrial protease supercomplex? Given that Yme1 and PARL have been shown by others (not necessarily from the Langer group) to effect Opa1 cleavage, it would be highly informative to test whether these proteases may comigrate with Opa1 on their gradients. The Scorrano group previously identified a role for PARL in the heat shock induced cleavage of Opa1 (Sanjuan et al. BBA 2012). Is this related to the Oma1 cleavage, or a distinct pathway. The authors have not mentioned PARL within this manuscript, but they have a great opportunity to address this point.

4. The authors do not incorporate the phenotype of the Oma1 KO mice into their discussion (Quiros et al., EMBO J 2012), and instead comment on the potential role of Oma1 in mitophagy, paralleling Opa1 cleavage with PINK1 accumulation and Parkin recruitment. Although CCCP may be an excellent tool to delineate a pathway, it remains unclear how often mitochondria lose potential in vivo. Notably Oma1 KO animals do not present obvious neurodegenerative defects. Similarly, the PINK1 and Parkin KO mice also do not have a degenerative phenotype, and one of the most robust phenotypes is that Parkin KO animals cannot gain weight on a high fat diet (Kim et al, JCI 2011). The point is that the authors should attempt to address the physiological role of Oma1 by considering the phenotypes of the null mice rather than only focusing on the effects of CCCP treatments in vitro.

5. The title explains that Oma1 autocatalytic cleavage regulates mitochondrial dynamics, however changes in mitochondrial dynamics were inferred/assumed, but not shown. Therefore the title should be changed. For example, should Oma1 have substrates other than Opa1, some of the mutants may show unexpected morphological or functional phenotypes.

Minor points:

1. It would be helpful if the authors would include a model of Oma1 inserted into the membrane in 5C as well as the linear depiction of the species variants. The amino acid numbers of the domains that are described would also be helpful when considering all the mutants in the figures.
2. The authors did not specifically describe which residues were mutated in their -1 to -6 constructs. The methods only describe that they used PCR to make point mutants, and should be more precise in the description of these reagents.

Referee #3

Baker et al. studied processing and activation of the human mitochondrial metallo-protease OMA1. The paper starts with several confirmatory experiments, but is then followed by a number of interesting new observations. The most exciting new point is evidence for activating and inhibitory sequences within Oma1 and for inactivation through autocatalytic cleavage. The authors first identify an unusual N-terminal processing site in Oma1. They then present evidence for regulatory sequences just behind this processing site. They have data suggesting that both N and C termini regulate Oma1 and that regulation occurs through autocatalytic proteolysis. Finally they show incorporation in a 300-kDa complex. The authors' new model suggests that OMA1 is normally kept inactive by auto-inhibitory sequences, but can then somehow be activated by electrostatic changes.

The results clearly show that N-terminal mutations can inhibit cleavage while C-terminal mutations promote cleavage, but it does not follow that the uncleaved protein is the active form. That conclusion is based on an antibody that does not detect endogenous protein. The data also clearly show that OMA1 can be in a 300 kDa complex but it was not shown that the protein only acts within this complex. Release could conceivably also promote cleavage of other proteins like Opa1.

Caution is needed, because of previous erroneous conclusions based on similar heterologous expression and overexpression approaches. Assuming, however, that the results obtained with overexpressed Oma1 faithfully reflect the properties of endogenous protein, then these experiments are interesting and the results present an important advance.

Some more specific issues:

1. Experiments in Fig. 1 are confirmatory. The authors show that OMA1 is activated by stressors, such as loss of membrane potential, and that OMA1 *-/-* cells can be rescued with transfected OMA1. There is one unexplained difference with published results, namely that loss of ATP through oligomycin can activate proteolysis in earlier reports but not here. Were the drugs used here effective?
2. The protease protection experiments in Fig. 2 are confirmatory, but the TEV experiment are useful because the S1 site was originally thought to be cleaved by a matrix protease and the topology of OMA1 was never properly established.
3. Evidence for clipping of OMA1 at both ends is confusing (fig. 5D-F). The antibody against Oma1 is poorly described so it is unclear what exactly is recognized. There is very little of the C-terminally clipped product and it is even possible that a proteolytically active fragment ran off the gel.
4. Inhibitory role of c-terminus is also unclear. There is substantially less OMA1 in cells expressing the C-terminal deletion. Maybe that is why the degradation of oma1 is slower. Alternatively, maybe the C-terminal mutant is not incorporated in the 300 kDa complex, which could make it more active towards Opa1 but not towards itself.
5. Small point: the oma1 antibody recognizes bands in the CCCP treated fractions in fig. 7. Is this transfected Oma1 that lost its tail or endogenous Oma1?
6. The Oma1 antibody does not detect endogenous protein; instead overexpressed protein is used throughout. Overexpression is most worrisome with the complementation experiment in Fig 7 B where a lot more protein is present in the complementing samples, while the overall activity is low (not much Opa1 is cleaved). Can the amounts be adjusted to show that equal levels cause complementation?
7. In that same experiment: does the complemented sample become CCCP sensitive? This would represent true complementation and make this experiment more convincing.

Response to reviewer 1:

Specific points:

1. As suggested by the reviewer, we monitored the activity of OMA1-myc upon mitochondrial depolarization (new Fig. 1B). This experiment is consistent with our previous findings upon expression of OMA1-myc in *Oma1*^{-/-} MEFs and demonstrates unambiguously the functionality of OMA1-myc.
2. To directly demonstrate assembly of OMA1E324Q-myc and OMA1 Δ 144-163-FLAG, we performed co-immunoprecipitation experiments using *hOma1*^{-/-} cells transiently expressing the mutant proteins (new Fig. S5). Immunoprecipitation with myc- or FLAG-specific antibodies resulted in the precipitation of both mutant proteins. Although the precipitation efficiency is limited, these experiments demonstrate a physical interaction of both mutant OMA1 variants.

Minor points:

- A) We now include a more extensive description for the establishment of *Oma1*^{-/-} HEK293 cells by zinc finger nucleases on pp. 6/7.
- B) We now label immunoblots with Myc (OMA1).
- C) We include a scheme of OPA1 splice variants 1 (L-OPA1 b) and 7 (L-OPA1 a) and the products of proteolytic processing (S-OPA1 c, d and e) as the new Fig. 1A. We also monitored mitochondrial fragmentation upon H₂O₂ treatment directly using immunofluorescence microscopy (new Figs. 1G and H). Mitochondrial fragmentation upon other stress treatments has been described previously in various publications (including for instance Ishihara et al., 2006, Griparic et al., 2007).
- D) As indicated in the Material and Method section, proteinase K was used at a concentration of 50 μ g/ml. The reduced levels of both Smac and OMA1-myc (but not Hsp60) upon protease treatment of purified mitochondria (Fig. 2A) indicate that the outer membrane was partially disrupted as now stated in the figure legend. This is a phenomenon frequently observed upon purification of mitochondria and we therefore routinely perform immunoblotting against various control proteins. The similar behavior of OMA1-myc and Smac that is different from matrix localized Hsp60 is consistent with OMA1-myc being exposed to the IMS. PHB2 is known to be relatively protease resistant, which is why we do not observe a similar reduction as for Smac. We now discuss briefly the reduced amount of OMA1-myc in protease-treated mitochondria in the legend to Fig. 2A.
- E) Endogenous levels of OMA1 appear to be very low and we indeed did not detect OMA1 in MEFs and HEK293 cells. However, OMA1 was detectable using both myc and OMA1-specific antisera upon overexpression of OMA1-myc in HEK293 cells (Figs. 3E, F). We have clarified this issue on pp. 17/18 to avoid misunderstandings.
- F) Expression of OMA1-myc was induced by incubating cells for 16 h in the presence of 1 μ g/ml tetracycline as indicated in the Material and Method section, p. 7.

Response to reviewer 2:

1. We performed a number of new experiments to better define the events triggering OMA1 activation under stress. As suggested by the reviewer, we quantified the membrane potential under stress using JC1 or TMRM staining of mitochondria (new Figs. 1D, S1). Mitochondria were found to be depolarized under various stress conditions as expected or as in part observed previously (e.g. Duvezin-Caubet et al., 2006; Ishihara et al., 2006; Baricault et al., 2007; Guillery et al., 2008). However, OMA1 activation is not strictly coupled to mitochondrial depolarization. Antimycin A treatment results in the loss of the membrane potential but does not activate OMA1. On the other hand, the membrane potential was maintained upon oligomycin treatment while OMA1 was at least slightly activated. Similarly, we observed that heat stress activates OMA1 without dissipating the membrane potential. How OMA1 is

activated under these conditions remains speculative but it is tempting to speculate that conformational changes of OMA1 are facilitated at increased temperatures. It thus appears that different molecular events may influence the conformation of OMA1 in the inner membrane and trigger its activation. Notably, ROS are apparently not required for OMA1 activation. In agreement with previous findings (Baricault et al., 2007), we did not observe any stabilization of L-OPA1 in depolarized mitochondria upon addition of N-acetylcysteine. We discuss these new findings on p. 27 of the manuscript.

2. We have analyzed the assembly of OMA1 variants including OMA1-6-myc by sucrose gradient centrifugation. As now shown in Fig. S5, all variants form similar-sized high molecular weight complexes in the inner membrane.

3. The apparent molecular mass of OMA1 oligomers suggests a hexameric structure, consistent with previous findings in yeast (Käser et al., 2003). However, reconstitution experiments will be required to confirm the oligomeric state of OMA1, which is beyond the scope of the present manuscript. Similarly, the presence of a protease supercomplex is an intriguing possibility that in general deserves further investigation. However, we feel that this question is not of direct relevance to the present study, as OMA1 activation is not accompanied by alterations in the oligomeric state of OMA1 (see point 2). With respect to the role of PARL, we would like to emphasize that various groups (including our own group) have published compelling evidence that PARL – though functionally linked to OPA1 - is not required for OPA1 processing (Duvezin-Caubet et al., 2006; Griparic et al., 2008).

4. We agree with the reviewer that the phenotypic characterization of the OMA1 knockout mice provides the most compelling (and so far only) insight into the physiological role of OMA1 *in vivo*. We have therefore mentioned this work in the introduction and now also revised the discussion on p. 27. Although mitochondrial depolarization induced by uncouplers *in vitro* allowed us to obtain mechanistic insight into OMA1 activation, this likely does not represent the most relevant stress condition *in vivo*, as pointed out by the reviewer. Regardless, we feel that it is likely of interest to the broad readership to point out the striking similarities of stress conditions that activate the PINK1/Parkin pathway at the outer membrane and OMA1 in the inner membrane *in vitro*.

5. We have now monitored directly the morphology of mitochondria under oxidative stress (Figs. 1G, H) and in the presence of different OMA1 variants (Figs. 6B, C). The latter experiments revealed an increased resistance of depolarized mitochondria against fragmentation, further substantiating the critical role of the N-terminal domain of OMA1 for the stress response. We therefore did not change the title of our manuscript.

Minor points:

1. We show a model for OMA1 topology in the new Fig. 2C and included numerals indicating the amino acid position of important domains in the schematic representation of OMA1 species variants in the new Fig. 5C.

2. We apologize for this mistake and now include the relevant information in the Material and Method section of our manuscript (p. 6/7).

Response to reviewer 3:

General comments:

We thank the reviewer for the overall positive evaluation of our work. We are fully aware of the experimental difficulties caused by the low expression level/instability of OMA1, making it difficult to detect the endogenous protein by immunoblot. However, we would like to emphasize that we use overexpression only to analyze the role of specific domains

in OMA1 and, in these experiments, always compare OMA1 variants with wild type OMA1 present at similar levels. Otherwise, we use knockout MEFs or HEK293 cells to examine the function of OMA1 in the response to mitochondrial stress.

Due to the intrinsic instability of OMA1 upon activation it is difficult to unambiguously exclude that a smaller form of OMA1 is active. However, it is important to note that mature OMA1 is active under normal conditions. Moreover, we did not observe the accumulation of N- or C-terminal OMA1 fragments under stress. Both findings suggest that mature but otherwise uncleaved OMA1 is proteolytically active. Similarly, OMA1 quantitatively assembles into a 300 kDa complex that is not altered upon dissipation of the mitochondrial membrane potential (as shown in Fig. 7A). This observation strongly suggests that the 300 kDa complex represents the proteolytically active form.

Specific comments:

1. We kindly disagree with the reviewer that the experiments in Fig. 1 are confirmatory. While induced OPA1 processing has been described under various stress conditions, a function of OMA1 has only been demonstrated in depolarized mitochondria (Head et al., 2009; Ehses et al., 2009; Quiros et al., 2012). The experiments shown in Fig. 1 now extend the role of OMA1 to other stresses including oxidative stress and heat stress. Oligomycin can indeed activate OMA1-mediated OPA1 processing as shown previously (e.g. Baricault et al., 2007; Ehses et al., 2009). We used shorter incubation times in our experiments to document the additive effect of oligomycin and antimycin A. However, as shown in the revised Fig. 1C, oligomycin induces OPA1 processing to some extent even under these conditions.
2. OMA1 was described to accumulate in a protease-protected form within mitochondria (Head et al., 2009), but the submitochondrial localization and the membrane topology of mammalian OMA1 has not been examined previously. Therefore, we consider the results in Figs. 2A and B as novel rather than confirmatory.
3. C-terminal proteolytic fragments of OMA1-myc are transiently formed upon mitochondrial depolarization indicating N-terminal processing (Figs. 3E, 4C, 5D, 5E). On the other hand, we observe the transient accumulation of an OMA1 fragment that is only slightly smaller than mature OMA1-myc but not recognized by myc-specific antibodies, indicating C-terminal processing. Proteolytic fragments of OMA1 do not accumulate stably upon prolonged incubation of depolarized mitochondria suggesting that OMA1 is degraded completely. The OMA1-specific antibody was raised against an OMA1 domain (amino acids 211-521) that was heterologously expressed in *E. coli*, as now indicated in the Material and Method section (p. 11).
4. The accelerated OPA1 processing by C-terminally truncated OMA1 (Fig. 5D) can be explained by an inhibitory role of the C-terminal region for OMA1 activity, reminiscent of distantly related metallopeptidases (Lopez-Pelegrin et al., 2013). We have discussed this hypothesis on p. 27 of the manuscript. However, we agree with the referee that the decreased autocatalytic turnover of OMA1 itself remains puzzling and may point to a role of the C-terminal region for autocatalytic turnover. While a definite answer to these questions will require insights into the structure of OMA1, deletion of C-terminal amino acid residues of OMA1 does not interfere with its assembly in the 300 kD complex as revealed by sucrose gradient centrifugation (now shown in Fig. S5).
5. HEK293 cells overexpressing OMA1-myc were used in the experiment shown in Fig. 7A. We have analyzed the oligomerization of OMA1 after short incubation with CCCP. The band recognized by the OMA1-specific antibodies represents a Cterminally truncated variant of OMA1 that lacks the myc-tag and transiently accumulates under these conditions (see also Fig. 3F).
6. It is difficult to adjust protein levels after transient transfection in the experiments in Fig. 7B. As immunoblot analysis only allows to examine OMA1 levels in the whole cell

population, expression levels in individual cells cannot be deduced from these experiments. OMA1 activity upon expression of both mutant variants is low, but the effect on OPA1 processing is highly specific. Even high overexpression of the proteolytically inactive variant OMA1E324Q or OMA1 Δ 144-163 does not induce OPA1 cleavage as shown in Figs. 4C, 5D, and 5E).

7. We have tested OPA1 processing upon CCCP-treatment of *hOma1*^{-/-} cells expressing both mutant variants as suggested by the reviewer. We did not observe an increased OPA1 processing upon mitochondrial depolarization (Fig. 7B). This is in agreement with an important role of the N-terminal sensor domain of OMA1 for activation rather than for proteolytic activity per se (p. 23).

2nd Editorial Decision

22 December 2013

I have now received comments (see below) from two of the original referees of your manuscript who are both satisfied with the amount of revisions and support publication.

I am thus pleased to accept your manuscript for publication in the EMBO Journal. Please see below for important information on how to proceed.

Thank you for contributing to the EMBO Journal!

Please note:

I would be grateful at this stage if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

REFeree COMMENTS

Referee #2:

The authors have addressed each of my concerns with new experiments, and adjustments within the text. I have no issues remaining as the data is of high quality and the study provides significantly more insight into the mechanisms and dynamics of OMA1. I look forward to future studies that continue to explore additional substrates and the physiological regulation of this protease in vivo.

Referee #3:

Baker et al. adequately addressed the comments by the reviewers and added some intriguing new data. In my mind they now have complete story. It is an excellent paper.