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Membrane Depolarization Activates the Mitochondrial Protease OMA1 By Stimulating Self-cleavage

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 22 December 2013

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that all reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they also point out aspects of your study that should be further strengthened before publication. For example, referees 2 and 3 raise concerns about the specificity of the antibody used to detect Oma1 cleavage products. It would strengthen that data if some of the experiments could be validated with another antibody against Oma1; if this is not possible, the specificity issue would need to be addressed in a different way. Referee 2 also points out that it should be tested whether cleaved Oma1 remains bound to the full length protein and that an explanation for the fact that Opa1 is cleaved even in the absence of Oma1 should be provided. Referee 3 feels that stronger proof for the claim that the 34 kD form of Oma1 (and not, for example, the 50 kD fragment) is indeed the active fragment is needed. This reviewer also indicates that an explanation for the effects of prohibitin depletion on Oma1-dependent Opa1 cleavage should be provided, as they are in contrast to previous findings. Referee 3 also states that the possibility that deletion of the autocatalytic cleavage site in Oma1 leads to inhibition of autocatalytic processing

due to protein misfolding should be addressed. Finally, reviewer 1 indicates that in some instances crucial controls and additional clarification would need to be provided.

Given the overall interest of your findings and the reviewers' constructive comments on how to improve the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 28,000 characters (including spaces and references). Please note that currently, your manuscript contains over 35,000 characters and thus would need to be shortened slightly. While you may consider displaying peripheral results as supplementary information, the materials and methods required for the understanding of the main experiments may not be displayed in the supplementary section only.

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REFEREE REPORTS:

Referee #1:

Mitochondrial morphology is constantly adapted to the physiological conditions of the cell. The mitochondrial dynamin-like protein OPA1 plays a key role in this process. OPA1 activity is modulated by processing at two sites, and the protease OMA1 is crucial for OPA1 processing. Here, Zhang et al. report that OMA1 activity is also dependent on processing, and that OMA1 is a self-processing peptidase. This is a significant key finding that is of interest to the molecular biology community. However, the following points should be addressed by the authors.

Major points

1. The blots shown in Fig. 3B should not only show the disappearance of L-OMA1 but also the appearance of S-OMA1. This figure lacks a control showing plasmid-borne expression of WT OMA1. Is WT OMA1 cleaved after transfection into OMA1-null mutants, or does OMA1 cleavage depend on preexisting OMA1?

2. Figure S2 should show some representative fluorescence micrographs. This is an important experiment that should appear in the main figures instead of the supplement.

3. Is the ectopic expression of S-OMA1 sufficient to induce OPA1 processing in the absence of

CCCP? And what is the effect on mitochondrial morphology? This is a straight-forward experiment to test the authors' model and could greatly strengthen the paper.

4. Grammar should be checked carefully. The results section is sometimes hard to understand and could profit from extensive rewriting. I have to admit that I had difficulties to follow some parts of the text even after reading it several times.

Further points

5. The experiment shown in Fig. 1B is not entirely clear. Is S-OMA1 generated by cleavage of OMA1-Flag or OMA1, or both? I guess it is an N-terminal fragment, as S-OMA-1 is not detected by anti-Flag in Fig. 2. This should be mentioned already here. Are there additional bands smaller than 34 kDa? The entire blot should be shown here. Molecular weight markers are lacking in Fig. 1C. Therefore it is difficult to compare the bands to Fig. 1B.

6. At least in my eyes S-OMA1 (lane 4) and OMA1_delta453-521 (lane 5) have the same size (Fig. S1).

7. OPA1 band 'e' is hardly visible in Fig. 3B and C. Band 'd' is much stronger than 'e' in Fig. 3B and C, whereas 'e' is much stronger than 'd' in Fig. 3D. Why is this?

8. The quantifications in Fig. 4B and C are confusing. I don't understand which lanes in the blots they correspond to.

9. The figures and their panels should appear in the order they are mentioned in the text, i.e. first 3A, then 3B etc.

Referee #2:

In this report the authors examine the mechanism of Oma1 regulation in the regulated cleavage of Opa1 during mitochondrial dysfunction. It had been shown that Oma1 became activated somehow upon mitochondrial uncoupling, and that the generation of short forms of Opa1 blocked mitochondrial fusion. In the current study the authors provide a new observation that Oma1 itself becomes cleaved in an autocatalytic manner upon loss of potential or of the prohibitins. Their data suggest that the cleaved form of Oma1 is required to promote the generation of short Opa1 forms, and therefore likely reflects the active form. The half-life of the cleaved Oma1 enzyme is relatively short, presumably limiting providing a window for recovery through new Oma1 import. The authors perform site-directed mutagenesis approaches to map the Oma1 cleavage site, and use a variety of cell lines, including Oma1 null MEFs to test their ideas. Overall the evidence that Oma1 is autocatalytically cleaved is important, but there are weaknesses in the data, which may confuse the interpretation.

1. If Oma1 is required to cleave Opa1 in the presence of CCCP, then why do we observe some cleavage in the Oma1 null cells shown in Figure 3B compare lanes 2 to 5, and again in 3C, lanes 1 and 2 (or at least loss of the upper bands)? Certainly overexpressing Oma1 leads to an increase in cleavage even without CCCP, which is further enhanced with depolarization. These experiments allow the dissection of the mutants of Oma1, but this first point was confusing.
2. The Oma1 antibody appears quite variable, as the null cells sometimes show a significant number of bands. For example, in 3C there is significant signal in the null cells when probed with anti-Oma1, which doesn't look that different from some of the mutants shown on the gel. In other figures the Oma1 antibody shows only one or two non-specific bands. The point of this story revolves around cleavage products revealed with this antibody, so the variability with the antibody is concerning. The loss of the flag epitope is consistent with the hypothesis, but together with my point 1, it becomes clear that all of this data would benefit from better quantification to control for these variables between gels and blots. In the cyclohexamide chase experiments there is some quantification, but no error bars are included as this is from a single experiment.
3. It would be important to determine whether the short form of Oma1 may remain bound to the long form. The assumption that the short is exclusively active to cleave Opa1 would indicate that it

acts alone. Some co-IPs would be informative here since they predict that the short form may relocalize within the mitochondria. As it remains anchored in the membrane, it is unclear how this might occur.
4. I recommend that the morphological quantifications found within the supplemental data should be brought into the primary figures since this is a critical functional output for Oma1.
5. The text could be improved for language issues throughout.

Referee #3:

Zhang et al. describe structure function studies of the OMA1 protease, which is responsible for CCCP induced OPA1 proteolysis. They show that OMA1 cleaves itself, using OMA1 -/- cells in which they transfect catalytic mutant or wildtype proteins. They identify a potential cleavage site within OMA1 and they show that OMA1 is further degraded in a manner that requires membrane potential and Prohibitin. The paper shows that endogenous precursor OMA1 is cleaved from a 60 kDa precursor to a relatively stable inactive pool of around 50 kDa for the FLAG tagged version or 40 kDa for endogenous protein. When treated with CCCP, OMA1 is further cleaved to a 34 kDa protein that can only be detected with the OMA1 antibody. This is proposed to be the active form. The observed cleavages are convincing, but the claim of an actual mechanism is overstated. There are some additional technical issues.
1. The conclusion that the 34 kDa fragment is the active form is not fully justified; the 50 kDa could be temporarily activated upon CCCP treatment and then inactivated by cleavage to the 34 kDa fragment. There must be a way to distinguish between these possibilities.
2. The effects of prohibitin are opposite to earlier results, which showed that prohibitin loss destabilizes L-OPA1. Those would suggest that Prohibitin inhibits Oma1 and so it is unclear why prohibitin knock down would stabilize the active form in the experiments presented here. This needs to be resolved.
3. Deletions of the autocatalytic cleavage site can lead to protein inactivation, but this result could also be explained as non specific interference with OMA1 function for example by misfolding of the protein, instead of selectively preventing the removal of an auto-inhibitory sequence. More definitive data is needed to support this. a
4. Fig. 1B and C shows a large number of bands, which are indicated as non-specific. They appear more strongly with CCCP. Importantly, they are recognized by FLAG and the OMA1 antibody, so there must be other intermediate, cleavage sites. How do the authors know that 34 kda is the relevant species?
5. 5 putative TM segments are indicated in Fig. S1. There is no experimental evidence for that many TMs.
6. The sizes are off quite a bit. Calculated MW for precursor is 60 kda, first cleavage, 50 Kda, second cleavage 41 kDa. Observed for endogenous: 60, 40 and 34 kda. This seems too much for aberrant mobility.

1st Revision - authors' response 03 February 2014

Response to Reviewer 1

Mitochondrial morphology is constantly adapted to the physiological conditions of the cell. The mitochondrial dynamin-like protein OPA1 plays a key role in this process. OPA1 activity is modulated by processing at two sites, and the protease OMA1 is crucial for OPA1 processing. Here, Zhang et al. report that OMA1 activity is also dependent on processing, and that OMA1 is a self-processing peptidase. This is a significant key finding that is of interest to the molecular biology community. However, the following points should be addressed by the authors.

Major points

1). The blots shown in Fig. 3B should not only show the disappearance of L-OMA1 but also the appearance of S-OMA1. This figure lacks a control showing plasmid-borne expression of WT OMA1. Is WT OMA1 cleaved after transfection into OMA1-null mutants, or does OMA1 cleavage depend on preexisting OMA1?

We appreciate the reviewer's comments and suggestions. Both L-OMA1 and S-OMA1 on the blot were showed and the control showing the expression of WT OMA1 was also added in Fig. 2B of revised manuscript (Fig. 3B in original manuscript).

To assess cleavage of OMA1, we subcloned mouse OMA1 cDNA (tagged with 3xFlag at C-terminal of OMA1) or its mutant OMA1-E324Q cDNA into retrovirus vector pMSCV-puro, the produced retrovirus were used to infect OMA1-null or WT MEFs cells, so WT or OMA1-null MEFs cells stably express WT OMA1 or mutant OMA1-E324Q protein; we observed that WT OMA1 but not OMA1-E324Q (without protease activity) is cleaved in OMA1-null cells upon CCCP treatment (Fig. 1F in revised manuscript), suggesting that the cleavage of exogenous expressed WT OMA1 by itself is not depend on preexisting OMA1 since there is no preexisting OMA1 in OMA1-null cells; additionally, the exogenous OMA1-E324Q is cleaved by the preexisting OMA1 (endogenous) because S-OMA1 was generated in HeLa cells (Fig. 1B in revised manuscript), suggesting that the preexisting OMA1 could cleave exogenous OMA1; concluded, OMA1 is cleaved by itself no matter whether OMA1 is exogenous expressed or is preexisting (endogenous).

2). Figure S2 should show some representative fluorescence micrographs. This is an important experiment that should appear in the main figures instead of the supplement.

According to the reviewer's suggestion, we show Fig. S2 of original manuscript (Fig. 3 in the revised manuscript) in the main figures instead of the supplement, and show the representative fluorescence micrographs in the Fig. 3A.

3). Is the ectopic expression of S-OMA1 sufficient to induce OPA1 processing in the absence of CCCP? And what is the effect on mitochondrial morphology? This is a straight-forward experiment to test the authors' model and could greatly strengthen the paper.

We totally agree with the reviewer's comment. We did express OMA1_delta443-521, OMA1_delta453-521 or OMA1_delta448-521 (similar with S-OMA1 in size) in OMA1-null MEFs cells to examine its effect on OPA1 processing and mitochondrial morphology in the absence of CCCP. We did not observe the ectopic expression of S-OMA1 has effect on OPA1 processing and mitochondrial morphology (data not show). We think direct ectopic expressed S-OMA1 may be dysfunctional. The de novo synthetic cytosolic WT OMA1 is need to be imported into mitochondrial inner membrane and be folded with the help of other mitochondrial proteins, and the conformation of L-OMA1 may be changed and cleaved to S-OMA1 to form functional conformation upon membrane depolarization; but direct ectopic expression of S-OMA1 lacking C-terminal may loss some interactions with other mitochondrial proteins and may not be folded rightly or cannot be imported into right place in mitochondria, therefore, ectopic S-OMA1 may be dysfunctional. Indeed, we observed the ectopic S-OMA1 is stable (data not show) but S-OMA1 generated by OMA1 cleavage is unstable and is quickly degraded (Fig. 4B in revised manuscript), indicating that the ectopic S-OMA1 has different conformation in comparison with the S-OMA1 generated by OMA1 cleavage. Additionally, the other cleaved short form C-terminal OMA1(C-OMA1) may help S-OMA1 to regulate OPA1 processing; due to the ectopic expression of C-OMA1 (without mitochondrial targeted sequence) cannot be imported into mitochondria, we cannot express exogenous S-OMA1 and C-OMA1 in mitochondria to check the effect of S-OMA1 on OPA1 processing and mitochondrial morphology. Taken together, we think that the activation of OMA1 is complicated, the functional S-OMA1 needs to be rightly folded and be in the right place in mitochondria under certain situation in cells, and S-OMA1 may need C-OMA1 to regulate OPA1 processing; so the direct ectopic expression of S-OMA1 in the cells without CCCP treatment is not enough to be functional. Finally, it will be interesting to note that the activation of OMA1 may be similar with caspases activation. Upon apoptotic stimuli, the procaspase-8 or procaspase-9 is cleaved by itself, the two cleaved forms interact with each other and form a complex which is functional, but the ectopic expression of cleaved caspase-8 or caspase-9 is dysfunctional since some other proteins (such as FADD for procaspase-8, or cytochrome C and Apaf-1 for procaspase-9) are also involved in the activation of caspase -8 or -9. About OMA1 activation, it still unknown whether some other factors are needed to activate OMA1.

To strength our model, WT MEFs cells or WT MEFs cells expressing OMA1-Flag were treated with CCCP to induce complete processing of L-OPA1, then cells were washed with PBS and continue to grow for indicated time, the processing of newly synthesized L-OPA1 were analyzed by

Western blot with anti-OPA1 antibody. As shown in Fig. S4A of revised manuscript, L-OPA1 is detectable at 2h after CCCP washout in WT cells (S-OMA1 is undetectable), however L-OPA1 appeared only at 4h after CCCP washout in the cells containing high level of S-OMA1, indicating that WT cells restored L-OPA1 more quickly than WT Cells with higher level of S-OMA1; in addition, S-OMA1 is still detectable at 2h after CCCP washout in WT cells expressing OMA1-Flag, suggesting that S-OMA1 is functional in L-OPA1 processing. To further examine the effect of S-OMA1 on L-OPA1 processing, we used Retro-X Tet-On Advanced Inducible Expression System (Clontech) to express OMA1-Flag in OMA1 null cells. With incubation of doxycycline (Dox; a tetracycline derivative), cells are induced to express OMA1-Flag (Figure S4-B in revised manuscript). After incubation with Dox for 48h, OMA1 null MEFs cells were turned on and expressed OMA1-Flag, cells were then treated with CCCP for 2h to induce S-OMA1 and remove L-OPA1 (completely processed); after CCCP washout, cells were incubated with Dox (lanes 3-6 in Fig. S4-C of revised manuscript), DMSO (lanes 7-10 in Fig. S4C of revised manuscript) or CCCP (lanes 11-14 in Fig. S4C of revised manuscript) for 2h, 4h, 6h or 8h, the cells lysates were checked for recovery of L-OPA1with Western blot. As shown in Fig. S4-C of revised manuscript, OMA1-Flag is recovered a little bit in lane 6 (incubation with Dox for 8h) but not recovered in lanes 7-14 due to the absence of Dox in cells, indicating that no OMA1-Flag was synthesized and only S-OMA1 to process de novo synthesized L-OPA1 in lanes 7-14. Comparing with lane 2, lanes 7-10 has increased OPA1 short form band ‘c’ (production is dependent on OMA1) (Fig. S4-C in revised manuscript), suggesting that S-OMA1 is functional in the processing of newly synthesized L-OPA1; additionally, as result of stabilization of S-OMA1 by CCCP treatment, little L-OPA1 is detectable in lanes 11-14 of Fig. S4-C of revised manuscript, indicating that de novo synthesized L-OPA1 is processed by S-OMA1, further confirmed that S-OMA1 has ability to process OPA1.

4). Grammar should be checked carefully. The results section is sometimes hard to understand and could profit from extensive rewriting. I have to admit that I had difficulties to follow some parts of the text even after reading it several times.

We appreciate the reviewer’s suggestions. We tried our best to revise our manuscript in English style and minor typos.

Further points

5). The experiment shown in Fig. 1B is not entirely clear. Is S-OMA1 generated by cleavage of OMA1-Flag or OMA1, or both? I guess it is an N-terminal fragment, as S-OMA-1 is not detected by anti-Flag in Fig. 2. This should be mentioned already here. Are there additional bands smaller than 34 kDa? The entire blot should be shown here. Molecular weight markers are lacking in Fig. 1C. Therefore it is difficult to compare the bands to Fig. 1B.

Thanks for bringing these points to our attention. As suggested by reviewer, we changed the Fig. 2A of original manuscript to be Fig. 1B in revised manuscript and Fig. 1B of original manuscript was changed to be Fig. 1C in revised manuscript. We totally agree the reviewer’s point that S-OMA1 is an N-terminal fragment, and S-OMA1 in Fig. 1B of revised manuscript is generated by cleavage of OMA1-Flag but not OMA1 because the expression of endogenous OMA1 is low and endogenous OMA1 cleaved form S-OMA1 is undetectable (Fig. 1B in revised manuscript). In addition, we showed the entire blot in Fig. 1C of revised manuscript (Fig. 1B of original manuscript) and didn’t detect any bands smaller than 34kD. According to the reviewer’s suggestion, molecular weight markers are added in Fig. 1D of revised manuscript (Fig. 1C of original manuscript).

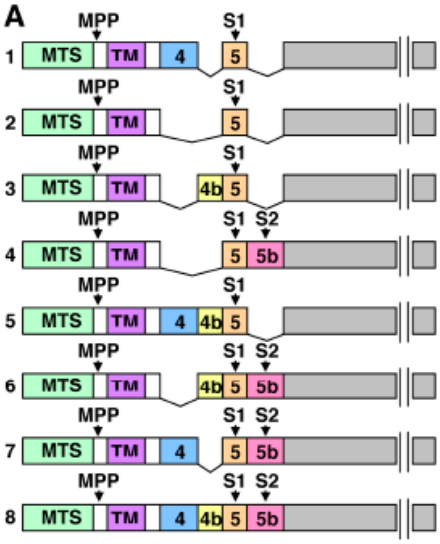
6). At least in my eyes S-OMA1 (lane 4) and OMA1_delta453-521 (lane 5) have the same size (Fig. S1).

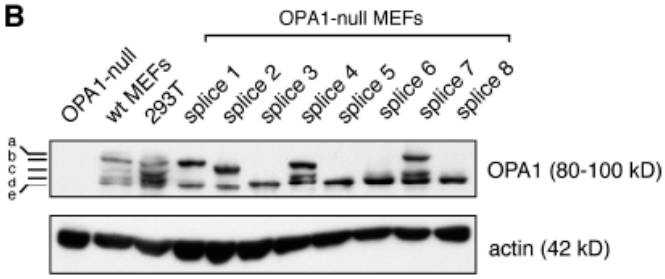
To strength data, we retested this experiment and the size of S-OMA1 was compared with OMA1_delta453-521 and OMA1-delta442-521. We think the size of OMA1_delta453-521 is a little bit larger than S-OMA1 in Fig. S1-B of revised manuscript.

7). OPA1 band 'e' is hardly visible in Fig. 3B and C. Band 'd' is much stronger than 'e' in Fig. 3B and C, whereas 'e' is much stronger than 'd' in Fig. 3D. Why is this?

Our previous data show that and OPA1 isoforms migrate as a complex mixture of five bands (a-e) on Western blot (Fig. A and B, shown as below) due to eight mRNA splice variants and subsequent proteolytic cleavage at S1 and S2 site of OPA1 (1). We pointed out that mitochondrial i-AAA protease Yme1L is responsible for OPA1 processing at S2 site (1), Thomas Langer Group and Alexander Van der Bleik Group reported that OMA1 mediates OPA1 cleavage at S1 site (2, 3). The

bands ‘c’ and ‘e’ are result from OPA1 processing at S1 site by mitochondrial protease OMA1, and band ‘d’ is generated by proteolytic cleavage at OPA1 S2 site by Yme1L (Fig. B, shown as below).



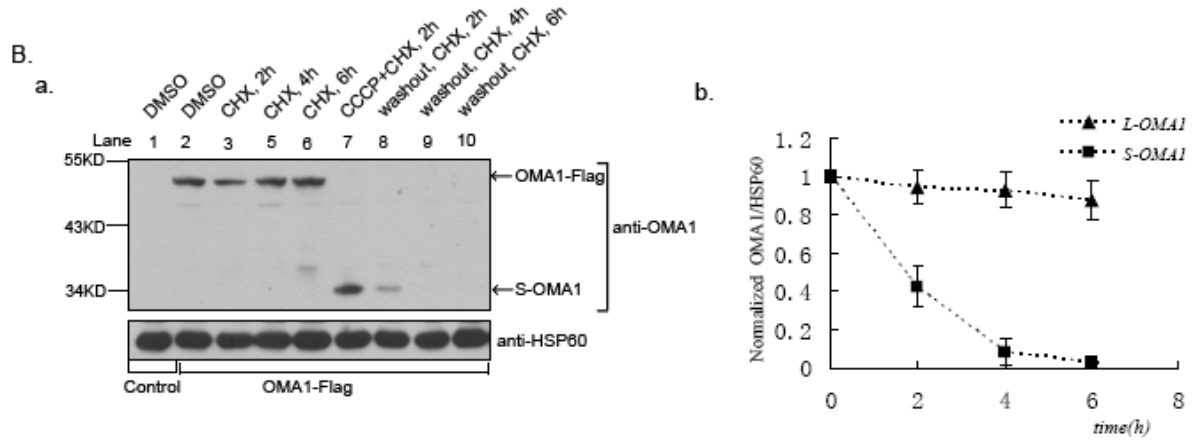


(Song *et.al*, 2007, ***J Cell Biol***)

In Fig. 2B and 2C of this revised manuscript (Fig. 3B and 3C of original manuscript), OPA1 short form ‘e’ is not generated in OMA1-null cells due to lacking S1 cleavage, and OMA1-delta445-450 or OMA1-delta443-452 in Fig. 2B of revised manuscript is dysfunctional towards OPA1 processing due to lacking S-OMA1 even in the presence of CCCP, and OMA1-delta445-448 or OMA1-delta447-450 in Fig. 2C of revised manuscript just maintains a little bit activity towards OPA1 cleavage at S1 site, so OPA1 band ‘e’ is hardly visible and band 'd' (generated by Yme1L) is much stronger than 'e' (produced by OMA1); additionally, in Fig. 2D of revised manuscript (Fig. 3D in original manuscript), some OMA1 point mutations (exogenous overexpressed) maintain much more activity (although their activity are less than WT OMA1) towards OPA1 processing than OMA1 mutations in Fig. 2B and 2C of revised manuscript, thus OPA1 protein band 'e' (generated by OMA1) is much stronger than 'd' in Fig. 2D of revised manuscript.

8). The quantifications in Fig. 4B and C are confusing. I don't understand which lanes in the blots they correspond to.

To show the difference of stability of L-OMA1 and S-OMA1 clearly, we used Image J software to analyze the intensity of protein bands L-OMA1(lanes 2-6), S-OMA1(lanes 7-10) and HSP60 (lanes 2-10) on the blot in Fig. 4B-a of revised manuscript (shown as below), and then calculated the ratio of L-OMA1/HSP60 and S-OMA1/HSP60, the ratios were then normalized, the ratio of L-OMA1/HSP60 (lane 2) and S-OMA1/HSP60 (lane 7) were normalized to ‘1’ (correspond to time ‘0’ in Fig. 4B-b, shown as below), time (h) ‘2’ in Fig. 4B-b corresponds to normalized L-OMA1/HSP60 (lane 3) and S-OMA1/HSP60 (lane 8), and time (h) ‘4’ in Fig. 4B-b corresponds to normalized L-OMA1/HSP60 (lane 4) and S-OMA1/HSP60 (lane 9).

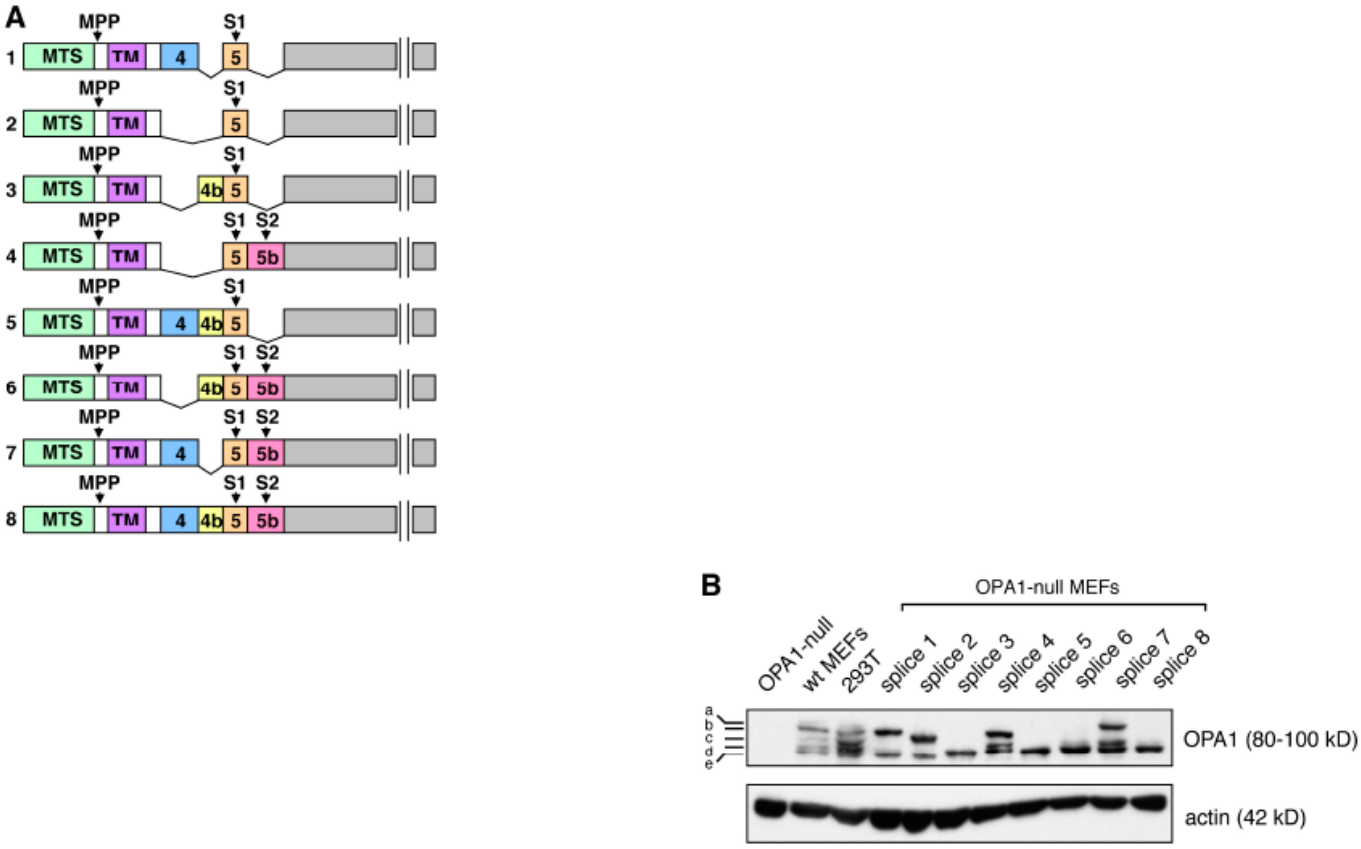


We also used the same method to quantify the intensity of S-OMA1 with or without CCCP (Fig. 4C-a in revised manuscript) and show the stability of S-OMA1 in the presence of CCCP or DMSO (Fig. 4C-b in revised manuscript).

9). The figures and their panels should appear in the order they are mentioned in the text, i.e. first 3A, then 3B etc.
Thanks for bringing this point to our attention. We show the figures and panels in the order in the text of our revised manuscript.

Response to Reviewer 2
In this report the authors examine the mechanism of Oma1 regulation in the regulated cleavage of Opa1 during mitochondrial dysfunction. It had been shown that Oma1 became activated somehow upon mitochondrial uncoupling, and that the generation of short forms of Opa1 blocked mitochondrial fusion. In the current study the authors provide a new observation that Oma1 itself becomes cleaved in an autocatalytic manner upon loss of potential or of the prohibitins. Their data suggest that the cleaved form of Oma1 is required to promote the generation of short Opa1 forms, and therefore likely reflects the active form. The half-life of the cleaved Oma1 enzyme is relatively short, presumably limiting providing a window for recovery through new Oma1 import. The authors perform site-directed mutagenesis approaches to map the Oma1 cleavage site, and use a variety of cell lines, including Oma1 null MEFs to test their ideas. Overall the evidence that Oma1 is autocatalytically cleaved is important, but there are weaknesses in the data, which may confuse the interpretation.

1). If Oma1 is required to cleave Opa1 in the presence of CCCP, then why do we observe some cleavage in the Oma1 null cells shown in Figure 3B compare lanes 2 to 5, and again in 3C, lanes 1 and 2 (or at least loss of the upper bands)? Certainly overexpressing Oma1 leads to an increase in cleavage even without CCCP, which is further enhanced with depolarization. These experiments allow the dissection of the mutants of Oma1, but this first point was confusing.
We appreciate the reviewer's comments and suggestions. OPA1 has eight mRNA splice variants (4). Our previous data show that OPA1 isoforms migrate as a complex mixture of five bands (a-e) on the blot (Figure A and B, shown as below) (1) due to eight mRNA splice forms and subsequent proteolytic cleavage. We showed that mitochondrial i-AAA protease Yme1L is responsible for OPA1 processing at S2 site (1), Alexander Van der Bleik Group and Thomas Langer Group reported that OMA1 regulates OPA1 cleavage at S1 site (2, 3). The bands 'a' and 'b' (Figure B, shown as below) are mixture of OPA1 long forms, the bands 'c' and 'e' (Figure B, shown as below) are result from OPA1 processing at S1 site by mitochondrial protease OMA1, and Band 'd' is generated by proteolytic cleavage at OPA1 S2 site by Yme1L.



(Song *et.al*, 2007, ***J Cell Biol***)

In OMA1 null cells, OPA1 is still cleaved at S2 site but not S1 site due to the presence of Yme1L, so band 'd' but not bands 'c' and 'e' were observed in lanes 2, 3, 6 and 8 in Fig. 2B of revised manuscript (correspond to lanes 2 to 5 in Fig. 3B of original manuscript) and lanes 1 and 2 in Fig. 2C of revised manuscript (Fig. 3B in original manuscript). Additionally, our previous data show that Yme1L is also associated with the degradation of OPA1 long forms (upper band 'a' and 'b') when cells is incubated with CCCP (5), so we can observe some loss of upper bands in lane 3 of Fig. 2B of revised manuscript (correspond to lane 5 in Fig. 3B of original manuscript) and in lane 2 of Fig. 2C of revised manuscript (Fig. 3C in original manuscript).

2). The Oma1 antibody appears quite variable, as the null cells sometimes show a significant number of bands. For example, in 3C there is significant signal in the null cells when probed with anti-Oma1, which doesn't look that different from some of the mutants shown on the gel. In other figures the Oma1 antibody shows only one or two non-specific bands. The point of this story revolves around cleavage products revealed with this antibody, so the variability with the antibody is concerning. The loss of the flag epitope is consistent with the hypothesis, but together with my point 1, it becomes clear that all of this data would benefit from better quantification to control for these variables between gels and blots. In the cyclohexamide chase experiments there is some quantification, but no error bars are included as this is from a single experiment.
Thanks for bringing these points to our attention. The OMA1 antibody we used is polyclonal, so there are some non-specific bands in the blots. We also tested some other commercial OMA1 antibodies, but the data are worse (data not shown). To improve the data, we blocked overnight and incubated the blots with anti-OMA1 antibody overnight for western blot analysis, then washed blots more times; as shown in Fig. 2B and 2C of revised manuscript (Fig. 3B and 3C in original manuscript), the data obtained by using anti-OMA1 antibody is better than before. Additionally, since OMA1 is located at mitochondria, so we used anti-OMA1 antibody to detect the purified mitochondrial extracts and obtained very good data since little no-specific bands were showed on the blot (Fig. 1D in the revised manuscript). To strength our data, a Myc-tag was inserted in the OMA1 protein (between OMA1 residue 101 and 102, as shown in Fig. S1A), we then analyze the processing of OMA1-Myc in the presence of CCCP with anti-Myc antibody. As shown in Fig. S1C

of revised manuscript, the data obtained by using anti-Myc antibody is very good, and OMA1-Myc was cleaved to a short form (S-OMA1-Myc) in cells treated with CCCP, further confirmed that OMA1 is cleaved upon membrane depolarization.

We also repeated cyclohexamide chase experiments two more times and further analyzed the data by quantification, and the error bars are included in Fig. 4B-b and 4C-b of revised manuscript.

3). It would be important to determine whether the short form of Oma1 may remain bound to the long form. The assumption that the short is exclusively active to cleave Opa1 would indicate that it acts alone. Some co-IPs would be informative here since they predict that the short form may relocalize within the mitochondria. As it remains anchored in the membrane, it is unclear how this might occur.

We appreciate the reviewer's suggestions. We performed co-IP experiments and showed that exogenous expressed OMA1-E324Q-Flag (long form) is able to precipitated endogenous OMA1 (long form) in HeLa cells (Fig. S4D-a in revised manuscript), suggesting that OMA1 long form binds to the long form. Upon membrane depolarization (CCCP treatment), exogenous OMA1-Flag and endogenous OMA1 long form disappeared and short form S-OMA1 emerged (lane 2 in Fig. S4D-b of revised manuscript) due to OMA1 processing, and OMA1-Flag long form failed to bind to short form S-OMA1 (lane 4 in Fig. S4D-b) since almost all long form is processed in the presence of CCCP, suggesting that S-OMA1 regulates OPA1 processing is independent on OMA1 long form when cells are membrane depolarized. Additionally, in CCCP treated HeLa cells expressing exogenous OMA1-E324Q-Flag, both OMA1 long form and short form exist (lane 2 in Fig. S4D-b) because a little bit active WT endogenous OMA1 is able to cleave part of OMA1-E324Q-Flag mutant to short form S-OMA1; as shown in lane 3 of Fig. S4D-b, OMA1-E324Q-Flag could precipitate OMA1 short form (S-OMA1), suggesting that short form of OMA1 remains ability to bind the long form. Since OMA1 long form could bind to and cleave another long form, the interaction between short form and long form may be the intermediate state of OMA1 long form self-cleavage. In addition, the interaction of OMA1 short form with long form indicates that the short form still locates at mitochondrial inner membrane, but whether OMA1 short form has changed conformation still need to be investigated in the future.

4). I recommend that the morphological quantifications found within the supplemental data should be brought into the primary figures since this is a critical functional output for Oma1.

Thanks for reviewer's suggestion. We show morphological quantifications (Fig. 2 in the revised manuscript) in the primary figures instead of the supplemental data in our revised manuscript.

5). The text could be improved for language issues throughout.

We appreciate the reviewer's suggestion. We tried our best to revise our manuscript in English style and minor typos.

Response to Reviewer 3

Zhang et al. describe structure function studies of the OMA1 protease, which is responsible for CCCP induced OPA1 proteolysis. They show that OMA1 cleaves itself, using OMA1 -/- cells in which they transfect catalytic mutant or wildtype proteins. They identify a potential cleavage site within OMA1 and they show that OMA1 is further degraded in a manner that requires membrane potential and Prohibitin. The paper shows that endogenous precursor OMA1 is cleaved from a 60 kDa precursor to a relatively stable inactive pool of around 50 kDa for the FLAG tagged version or 40 kDa for endogenous protein. When treated with CCCP, OMA1 is further cleaved to a 34 kDa protein that can only be detected with the OMA1 antibody. This is proposed to be the active form. The observed cleavages are convincing, but the claim of an actual mechanism is overstated. There are some additional technical issues.

1). The conclusion that the 34 kDa fragment is the active form is not fully justified; the 50 kDa could be temporarily activated upon CCCP treatment and then inactivated by cleavage to the 34 kDa fragment. There must be a way to distinguish between these possibilities.

We appreciate the reviewer's comments and suggestions. It is a high possibility that S-OMA1 (34 kDa fragment) is the active form. Firstly, in revised Fig. 4B-a, $OMA1^{-/-}$ cells expressing OMA1-Flag were treated with CCCP plus CHX to generate S-OMA1, after CCCP washout, S-OMA1 declined to about 1/5 after CHX treatment for 2 hours and disappeared when cells were incubated

with CHX for 4 hours (revised Fig. 4B), revealing that S-OMA1 is unstable protein and degrades completely within 4 hours; additionally, S-OMA1 degrades more quickly than S-OMA1-E324Q even in the presence of CCCP in revised Fig. S3B, suggesting that S-OMA1 is active for its own degradation since no L-OMA1 was synthesized in the cells treated with CHX. Secondly, to further check the effect of S-OMA1 on L-OPA1 processing, we used Retro-X Tet-On Advanced Inducible Expression System (Clontech) to express OMA1-Flag in OMA1 null cells. With incubation of doxycycline (Dox; a tetracycline derivative), cells are induced to express OMA1-Flag (Fig. S4B in revised manuscript), without Dox, the expression of OMA1-Flag is shut down but other endogenous proteins such as OPA1 are still expressing. After incubation with Dox for 48h, $OMA1^{-/-}$ MEFs cells were turned on to expressed OMA1-Flag, cells were then washed and treated with CCCP for 2h to induce S-OMA1 and remove L-OPA1 (completely processed); after CCCP washout, cells were incubated with Dox (lanes 3-6 in Fig. S4C of revised manuscript), DMSO (lanes 7-10 in Fig. S4C of revised manuscript) or CCCP (lanes 11-14 in Fig. S4C of revised manuscript) for 2h, 4h, 6h or 8h, the cells lysates were checked for recovery of L-OPA1 by Western blot. As shown in Fig. S4C of revised manuscript, L-OMA1 is recovered a little bit in lane 6 (incubation with Dox for 8h) but is not recovered in lanes 7-14 due to the absence of Dox in cells, indicating that no new L-OMA1 was synthesized and only S-OMA1 to process de novo synthesized L-OPA1 in lanes 7-14. Comparing with lane 2, lanes 7-10 has increased OPA1 short form band 'c' (production is dependent on OMA1) (Fig. S4C in revised manuscript), suggesting that S-OMA1 is functional in the processing of de novo synthesized L-OPA1; additionally, as result of stabilization of S-OMA1 by CCCP treatment, little L-OPA1 is detectable in lanes 11-14 of revised Fig. S4C, indicating that de novo synthesized L-OPA1 is processed by S-OMA1, further confirmed that S-OMA1 has ability to process OPA1. It should be noted that the activity of S-OMA1 on OPA1 processing is different from the activity on S-OMA1 degradation since the processing of OPA1 is promoted but S-OMA1 is stabilized in cells treated with CCCP.

2. The effects of prohibitin are opposite to earlier results, which showed that prohibitin loss destabilizes L-OPA1. Those would suggest that Prohibitin inhibits Oma1 and so it is unclear why prohibitin knock down would stabilize the active form in the experiments presented here. This needs to be resolved.

Thomas Langer group showed that deletion of Prohibitin 2 (prohibitin is also lost) causes the loss of L-OPA1 and increased OPA1 short forms (6), we agree the reviewer's point that Prohibitin inhibits OMA1, in the other words, the loss of Prohibitin activates OMA1. In this manuscript, we showed that Prohibitin 1 or 2 knockdown results in the decrease of OMA1 long form (OMA1 long form is cleaved by itself when the OMA1 is activated) and Prohibitin knockdown stabilizes S-OMA1 (Fig. 4D), indicating that Prohibitin knockdown leads to increased S-OMA1 and increased OMA1 activity in cells, so we think our results in this manuscript are consistent with previous reports.

3. Deletions of the autocatalytic cleavage site can lead to protein inactivation, but this result could also be explained as non specific interference with OMA1 function for example by misfolding of the protein, instead of selectively preventing the removal of an auto-inhibitory sequence. More definitive data is needed to support this.

We agree the reviewer's point that deletion of cleavage site may result in the misfolding of the protein. To exclude this possibility, the crystal structures of protein OMA1 and mutant (deletion of cleavage site) need to be showed; we are cooperating with Dr. Chao Qi who is a good researcher in the field of protein X-ray crystallography and want to obtain the crystal structure of OMA1 protein, but it may take at least 2 years to get OMA1's crystal structure, we will show the data in our future manuscript. To support our conclusion, besides OMA1(Δ443-452)-Flag, a series of OMA1 mutants (deletion of less residues or point mutations indicated in Fig. 2B, 2C and 2D of revised manuscript) such as OMA1(Δ445-450)-Flag, OMA1(Δ445-448)-Flag, OMA1(Δ447-450)-Flag, OMA1(DR446-447AA)-Flag, OMA1(L445A)-Flag, OMA1(R447A)-Flag, OMA1(L448A)-Flag, OMA1(I449A)-Flag, OMA1(P450A)-Flag or OMA1(Δ449-450)-Flag were expressed in $OMA1^{-/-}$ MEFs cells separately, and cells expressing OMA1 mutant were treated with CCCP to check the processing of OMA1 and OPA1. As shown in Fig. 2B, 2C and 2D of revised manuscript, the extents of OMA1 processing is positively correlated with its activity towards OPA1 cleavage, suggesting that the less of OMA1 auto-cleavage (less S-OMA1 is generated), the less of L-OPA1 is processed, further confirmed that the processing of OMA1 is required for OPA1 processing at S1 site.

4. Fig. 1B and C shows a large number of bands, which are indicated as non-specific. They appear more strongly with CCCP. Importantly, they are recognized by FLAG and the OMA1 antibody, so

there must be other intermediate, cleavage sites. How do the authors know that 34 kda is the relevant species?

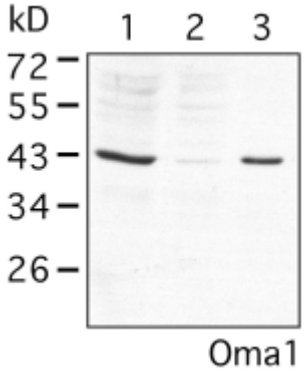
The OMA1 antibody we used is polyclonal, so there are some non-specific bands on the blots. We also tested some other commercial OMA1 antibodies, but the data are worse (data not shown). To improve the data, we blocked the blots overnight and incubated the blots with anti-OMA1 antibody overnight, then wash blots more times; as shown in Fig. 2B and 2C of revised manuscript, the data obtained by using anti-OMA1 antibody is better than before, 34kDa was clearly showed in the CCCP treated cells extracts but not in the control. In addition, since OMA1 is located at mitochondria, so we used anti-OMA1 antibody to detect the purified mitochondrial extracts, as shown in Fig. 1D of revised manuscript (Fig. 1C in original manuscript), we obtained very good data since little no-specific bands were showed on the blot, importantly, only 34kDa band was detected in the CCCP or Valinomycin treated mitochondrial extracts in Fig. 1D of revised manuscript. Additionally, in Fig. 1B and 1C of original manuscript, we didn't use anti-Flag antibody to dectect OMA1-Flag, we did use anti-Flag antibody to check OMA1 processing in Fig. 1B and 1E of revised manuscript (Fig. 2A and 2B in original manuscript), we didn't observe any intermediate bands on the blots detected by Western blot using anti-Flag antibody, and little non-specific bands were observed on the blots. We concluded that OMA1 is cleaved to S-OMA1 (34kDa) in the cells treated with CCCP.

5. 5 putative TM segments are indicated in Fig. S1. There is no experimental evidence for that many TMs.

Thanks so much for bringing this point to our attention. We agree the reviewer's point that the predicted TM segments by TMpred program are not fully convincing. Considering the limitation of journal (*Revised manuscripts, however, should be up to approximately* ***25,000 characters in length*** *including title page, abstract, references, figure legends and spaces (but excluding tables and Supplementary Information). ... In general,* ***display items (figures and tables) should be limited to 4*** *and the total length of the published paper should be between* ***6 and 7 pages*** *of the journal. ... In general,* ***Supplementary Information should be kept to an absolute minimum****, as appropriate to a short-format report*) and the data about TM segments are not key points in this manuscript, we removed the schematic for TM regions in Fig. S1A of revised manuscript. Since TM segments may play a very important role in OMA1 function, we are doing some experiments to check the accuracy of predicted TM segments, and will show TM regions and their roles in OMA1 function in our future manuscript.

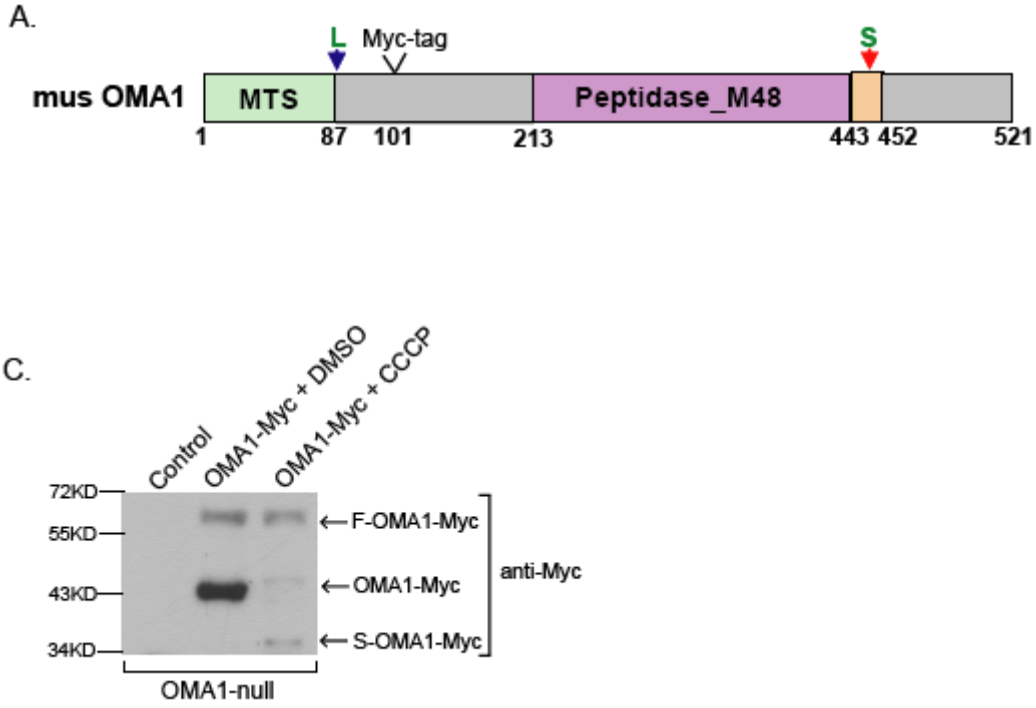
6. The sizes are off quite a bit. Calculated MW for precursor is 60 kda, first cleavage, 50 Kda, second cleavage 41 kDa. Observed for endogenous: 60, 40 and 34 kda. This seems too much for aberrant mobility.

Thanks so much for bringing this point to our attention. We agree the reviewer's point that the calculated MW for precursor of OMA1 (F-OMA1) is about 60 kDa, for OMA1 (the product of first cleavage) is about 50 kDa, for S-OMA1 (the product of second cleavage) is about 40 kDa, but we observed that MW for F-OMA1-Flag (Fig. 1B and S2 in revised manuscript) is about 60 kDa, for endogenous OMA1 is about 40 kDa (Fig. 1B and 1D in revised manuscript) and for endogenous S-OMA1 is about 34kDa (Fig. 1B and 1D in revised manuscript). We still felt puzzle about OMA1 MW at the beginning, we read the paper published by Van der Bliek group, they also showed that the MW for OMA1 is about 40 kDa (the figure is shown as below).



(Figure 1B, Head *et.al*, 2009, ***J Cell Biol***)

To further examine whether OMA1 has some other cleavage sites at N-terminal, we insert a Myc-tag in the OMA1 protein (between OMA1 residue 101 and 102, as shown in Fig. S1-A of revised manuscript), and we observed that the MW for OMA1-Myc about 44kDa (Fig. S1C in revised manuscript) which is consistent with the size for endogenous OMA1 (about 40kDa), indicating that the MW for endogenous OMA1 is different from the predicted size. Since the MW of OMA1 is about 40kDa, it is normal that the fragment of second cleavage is about 34kDa.



(Figure S1 in revised manuscript)

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2. Ehses S, Raschke I, Mancuso G, Bernacchia A, Geimer S, Tondera D, Martinou JC, Westermann B, Rugarli EI, Langer T. (2009) Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. *J Cell Biol.* 187(7):1023-36.
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2nd Editorial Decision 28 February 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once you have addressed the minor issues still raised by the referees with regard to some further clarifications and the inclusion of quantifications and issues with the representation of some of the results.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

The authors responded to most of my previous concerns in a satisfactory manner. Two remaining minor points should be addressed.

1. Scale bars are lacking for the fluorescence micrographs in the new Fig. 3. As there is only a single fluorescent label I recommend to show black and white images, instead of black and green, to improve the contrast between mitochondria and background.

2. The authors should mention somewhere in the manuscript that for unknown reasons ectopically expressed S-OMA1 was not sufficient to induce OPA1 processing in the absence of CCCP. As this is an obvious experiment the authors should mention the result, even if it is negative.

Referee #2:

My primary concerns were with the quality of the blots, with many background bands for Oma1, as well as a lack of quantification of some of the data. The authors have addressed these points and been more stringent with their blotting protocols to clean up the background. They included the functional imaging data into the main figures as well.

Overall, the characterization of the cleavage properties of Oma1 is an important advance for the field. It will have a high impact for those working in mitochondrial dynamics.

Referee #3:

Zhang et al. made improvements to their manuscript. I now find the notion that you need S-OMA1 for Opa1 cleavage convincingly shown. There is still uncertainty about site of the activating cleavage of Oma1 (around residue 450) and the effects of mutations, but I agree that a more definitive conclusion would require much more evidence than can be expected here.

Minor points:

1. Band e is hard to see in fig. 1. The authors argue in their rebuttal about this, but it really is just a matter of running better gels. I, nevertheless, don't think it is all that critical, since there is another

measure for cleavage: loss of band b. That works for most panels, except in panel B, last few lanes, where band b disappears with CCCP even when OMA1 is not cleaved and the cleavage site was deleted. What does that mean? There is still activation but no S-OMA1?

2. You also don't always see endogenous L- or S-OMA1, when you do expect it (wt cells in Fig. 2.B), but I guess that is a problem with amounts.

3. Legend for Fig. 2B talks about treating lysates with CCCP. Surely the cells were treated with CCCP before lysis, or else mitochondria were isolated and then treated.

4. Stabilization of S-OMA1 by CCCP in Fig. 4C seems pretty clear, but the stabilization by prohibitin shRNA (Fig. 4D) is not very clear. Without quantification and statistics, this remains to be seen.

5. The paper is still hard to follow. Especially towards the end, I was lost.

2nd Revision - authors' response 05 March 2014

Response to referee #1

The authors responded to most of my previous concerns in a satisfactory manner. Two remaining minor points should be addressed.

1. Scale bars are lacking for the fluorescence micrographs in the new Fig. 3. As there is only a single fluorescent label I recommend to show black and white images, instead of black and green, to improve the contrast between mitochondria and background.

We appreciate the referee's comments and suggestions. We have added the scale bars in the fluorescence micrographs in the new Fig. 3 of our new manuscript; we also change "black and green" images to "black and white" images in the new Fig. 3.

2. The authors should mention somewhere in the manuscript that for unknown reasons ectopically expressed S-OMA1 was not sufficient to induce OPA1 processing in the absence of CCCP. As this is an obvious experiment the authors should mention the result, even if it is negative.

According to the referee's suggestion, we discussed this result in our new manuscript, the added sentences in the text are attached bellow:

"*We also direct expressed S-OMA1 (OMA1_delta443-521, OMA1_delta453-521 or OMA1_delta448-521 which is similar with S-OMA1 in size) in OMA1$^{-/-}$ MEFs cells, we did not observe any effects on OPA1 processing and mitochondrial morphology, suggesting that ectopically expressed S-OMA1 alone was not sufficient to induce OPA1 processing for unknown reasons*".

Response to referee #2

My primary concerns were with the quality of the blots, with many background bands for Oma1, as well as a lack of quantification of some of the data. The authors have addressed these points and been more stringent with their blotting protocols to clean up the background. They included the functional imaging data into the main figures as well.

Overall, the characterization of the cleavage properties of Oma1 is an important advance for the field. It will have a high impact for those working in mitochondrial dynamics.

We appreciate the referee's comments to our manuscript.

Response to referee #3

Zhang et al. made improvements to their manuscript. I now find the notion that you need S-OMA1 for Opa1 cleavage convincingly shown. There is still uncertainty about site of the activating cleavage of Oma1 (around residue 450) and the effects of mutations, but I agree that a more definitive conclusion would require much more evidence than can be expected here.

Minor points:

1. Band e is hard to see in fig. 1. The authors argue in their rebuttal about this, but it really is just a matter of running better gels. I, nevertheless, don't think it is all that critical, since there is another measure for cleavage: loss of band b. That works for most panels, except in panel B, last few lanes,

where band b disappears with CCCP even when OMA1 is not cleaved and the cleavage site was deleted. What does that mean? There is still activation but no S-OMA1?
We appreciate the reviewer's comments and suggestions. The mitochondrial proteases Yme1L and OMA1 cleave OPA1 at S2 and S1 sites, respectively. We agree the referee's views that there are at least two measures for OPA1 cleavage by OMA1: loss of OPA1 long form band (band 'a' or 'b') or production of band 'c' and 'e'. Here, in some lanes of Fig.2B, OPA1 band 'b' disappears with CCCP even when OMA1 is not cleaved and the cleavage site was deleted, we think that OMA1 is not activated because no OPA1 band 'c' and 'e' was produced, the disappearance of OPA1 band 'b' is due to the degradation but not cleavage because Yme1L could degrade OPA1 band 'b' in the presence of CCCP (reference 1, shown as below). So, we still can observe a slight disappearance of OPA1 band 'b' even in OMA1$^{-/-}$ MEFs treated with CCCP (Fig.2C, lane 2).

2. You also don't always see endogenous L- or S-OMA1, when you do expect it (wt cells in Fig. 2.B), but I guess that is a problem with amounts.
The endogenous expression of OMA1 is different in different cell types. HeLa and 293 cells have high level of endogenous OMA1 protein but MEFs contains very low level of endogenous OMA1, so we don't see endogenous L- or S-OMA1 in WT MEFs cells (Fig.2B).

3. Legend for Fig. 2B talks about treating lysates with CCCP. Surely the cells were treated with CCCP before lysis, or else mitochondria were isolated and then treated.
Thanks so much for bringing this point to our attention. We incubated cells with CCCP and then treated cells with lysis buffer to obtain protein samples for Western blot. We have modified our legend for Fig.2B.

4. Stabilization of S-OMA1 by CCCP in Fig. 4C seems pretty clear, but the stabilization by prohibitin shRNA (Fig. 4D) is not very clear. Without quantification and statistics, this remains to be seen.
According to the referee's suggestion, we added the data (Fig.4D-b) for quantification and statistics in Fig. 4D in our new manuscript.

Reference

7. Ruan Y, Li H, Zhang K, Jian F, Tang J, Song Z. (2013) Loss of Yme1L perturbates mitochondrial dynamics. *Cell Death Dis*. 4: e896.

3rd Editorial Decision 06 March 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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