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SENP3-mediated DeSUMOylation of dynamin-related protein 1 Promotes Cell Death Following Ischemia

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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: Karin Dumstrei

1st Editorial Decision 04 February 2013

Thank you for submitting your manuscript to the EMBO Journal. This submission is a transfer from another journal with referee reports. Your manuscript has now been re-reviewed by two of the original referees. As you can see below, both referees appreciate the introduced changes and find this version significantly improved. I would therefore like to invite you to submit a revised version of the manuscript. There are still a few remaining comments that should be addressed, but it shouldn't involve too much additional work to resolve these. Regarding referee #2's comment to remove the Cathepsin B data, I find that dataset insightful, maybe you could slight soften the conclusions? We can discuss this issue further.

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

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

REFEREE REPORTS

Referee #1

Guo and colleagues have extensively revised this study in response to reviewer comments from an earlier submission to another journal. In fact, they have responded substantively to the reviewers' comments (including my own), providing additional and/or more convincing data as well as additional discussion.

Overall this study provides a large amount of data in support of the main points, and is clearly improved over the earlier version. Though multiple different post-translational modifications have now been shown to regulate the cellular distribution and/or function of Drp1, the alterations in SENP levels and downstream effects in response to OGD are clearly of great interest.

A few comments, mostly minor:

1. Can the authors propose a mechanism for the release of cytochrome c but not Smac/Diablo in response to Drp1 deSUMOylation? The data seem convincing, but it is not clear to me how mitochondrial fission would result in a selective release of cytochrome c over other IMS proteins.
2. Several of the references are incomplete.

Referee #2

The revised manuscript has been significantly improved, in particular the main section concerning the mechanism by which SENP3 triggers apoptosis through desumoylation of Drp1, mitochondrial fission and cytochrome c release. I still find the section on the role of Cathepsin B in SENP3 degradation not fully convincing. For example, the authors cannot guarantee that Cathepsin B is the protease responsible for SENP3 degradation in neurons. Therefore, I would suggest that the authors remove this part.
There are a number of comments that should be addressed:

1-In figure 3 C, I don't understand why Smac/DIABLO is released from mitochondria in cells transfected with an empty vector (pcDNA). Smac/DIABLO should remain within mitochondria in healthy cells. Is this a technical problem due to a suboptimal cytosol preparation, leading to damaged mitochondria?

2-The authors have excluded Bax as being responsible for cytochrome c release in cells overexpressing SENP3. What about Bak?

3-Figure 6E: Overexpression of Drp1 4KR leads to a strong LDH release (Figure 6E). How do the authors explain that the effect is weaker when Drp 4KR is expressed in SENP3 depleted cells (Figure 6F)?

4- The amount of LDH released from cells varies a lot according to the different experiments, sometimes performed in the same cells (Hek cells). What is the unit used for LDH activity and is there a way to homogenize the data.

1st Revision - authors' response 19 February 2013

Referee #1

Guo and colleagues have extensively revised this study in response to reviewer comments from an earlier submission to another journal. In fact, they have responded substantively to the reviewers' comments (including my own), providing additional and/or more convincing data as well as additional discussion.

Overall this study provides a large amount of data in support of the main points, and is clearly improved over the earlier version. Though multiple different post-translational modifications have now been shown to regulate the cellular distribution and/or function of Drp1, the alterations in SENP levels and downstream effects in response to OGD are clearly of great interest.

We thank Referee #1 for his/her positive comments.

A few comments, mostly minor:

1. Can the authors propose a mechanism for the release of cytochrome c but not Smac/Diablo in

response to Drp1 deSUMOylation? The data seem convincing, but it is not clear to me how mitochondrial fission would result in a selective release of cytochrome c over other IMS proteins.

We are pleased that the referee agrees that the data convincingly demonstrate that SUMOylation of Drp1 regulates the release of cytochrome *c*. What we can say is this is not via a mechanism that involves activation of MOMP because IMS proteins are not released. This is consistent with previous reports dissociating Bax/Bak-mediated MOMP from cytochrome c release in Drp1$^{-/-}$ MEFs (Ishihara *et al*, 2009) or Drp1 knockdown HeLa cells (Otera *et al*, 2010).

Our data suggest that SENP3 mediated deSUMOylation of Drp1 leads cytochrome *c* release via increased mitochondrial fission. We do not yet have a clear idea of the mechanism that allows this to be selective for cytochrome *c* compared to Smac/Diablo. This will be the focus of future work but we speculate that it may involve SUMOylation regulating Drp1 interaction with the mitochondrial anionic phospholipid cardiolipin (Montessuit *et al*, 2010).

2. Several of the references are incomplete.

We apologize for these errors and they have been corrected in the revised ms.

Referee #2

The revised manuscript has been significantly improved, in particular the main section concerning the mechanism by which SENP3 triggers apoptosis through desumoylation of Drp1, mitochondrial fission and cytochrome c release. I still find the section on the role of Cathepsin B in SENP3 degradation not fully convincing. For example, the authors cannot guarantee that Cathepsin B is the protease responsible for SENP3 degradation in neurons. Therefore, I would suggest that the authors remove this part.

We thank Referee #2 for his/her comments and we have further revised the manuscript to take account of the points raised.

We show that SENP3 is readily degraded by cathepsin B in vitro and by OGD in control MEF cells but not in cathepsin B$^{-/-}$ MEF cells. We interpret these findings to suggest that cathespin B participates in OGD-induced SENP3 degradation. Nonetheless, we were unable to prevent OGD-induced SENP3 loss in primary neuronal cultures with cathepsin B inhibitor II. Thus, we propose that there are also other pathways present in neurons that can degrade SENP3 during OGD.

We have revised the manuscript to make this clearer.

There are a number of comments that should be addressed:

1-In figure 3 C, I don't understand why Smac/DIABLO is released from mitochondria in cells transfected with an empty vector (pcDNA). Smac/DIABLO should remain within mitochondria in healthy cells. Is this a technical problem due to a suboptimal cytosol preparation, leading to damaged mitochondria?

We prepared samples using the ProteoExtract® Cytosol/Mitochondria Fractionation Kit (Calbiochem) according to the manufacturer's instructions. While we cannot fully exclude the possibility that some damage occurred to mitochondria during the protocol we detected only minimal levels of cytochrome *c* in the cytosol in cells transfected with an empty vector (*pcDNA3*). Initially, we also only detected very faint bands using Smac/DIABLO antibody (V-17, Santa Cruz biotechology). However, to for direct comparison between Smac/DIABLO levels between the four lanes, we used a high concentration of primary antibody (1:500) plus long exposure time to film.

To avoid any potential misunderstanding, we have repeated this experiment and replaced the figure with another blot using a lower concentration of primary antibody and shortening exposure time (see the revised Figure 3C).

2-The authors have excluded Bax as being responsible for cytochrome c release in cells overexpressing SENP3. What about Bak?

It has been reported previously that cortical neurons only express a BH3-only isoform of Bak (N-Bak), which is incapable of independently inducing mitochondrial dysfunction, cytochrome-*c* release and cell death (Uo et al., 2005). Thus, Bax is required in neuronal apoptosis.

Nonetheless, to fully exclude the possibility that Bak might play some role in SENP3-mediated we also tested if Bak activation is required for SENP3-mediated cytochrome *c* release in HeLa cells, which do possess full length Bak. As shown in the revised Figure 5A, STS treatment effectively activates Bak in HeLa cells but overexpressing either GFP-SENP3 or GFP-SENP3 C532A does not elicit any detectable Bak activation.

3-Figure 6E: Overexpression of Drp1 4KR leads to a strong LDH release (Figure 6E). How do the authors explain that the effect is weaker when Drp 4KR is expressed in SENP3 depleted cells (Figure 6F)?

As shown in Figure 6F, SENP3 knockdown is protective in cells in endogenous Drp1 was depleted and replaced with YFP-Drp1^{R} WT but it failed to protect in cells expressing YFP-Drp1^{R} 4KR. No comparisons of LDH release were made between cells in which endogenous Drp1 was replaced with Drp1^{R} WT or Drp1^{R} 4KR. We have now modified Figure 6F to make this clearer.

4- The amount of LDH released from cells varies a lot according to the different experiments, sometimes performed in the same cells (Hek cells). What is the unit used for LDH activity and is there a way to homogenize the data.

LDH levels were measured using the In Vitro Toxicology Assay Kit (Lactic Dehydrogenase Based; Sigma). This assay compares the relative levels of LDH between samples rather than absolute LDH values. See the following Sigma website for full details:

(http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/tox7bul.Par.0001.File.tmp/tox7bul.pdf).

In Figures 6B and 6C we monitored dynamic changes in LDH by presenting each value normalized to the 2h control value (mean ± SEM). In Figures 6D, 6E and 6F to allow for simple comparison, each LDH value is normalized to the corresponding control. SENP3 knockdown decreased LDH release by ~44%in Fig. 6B, ~35% in Fig. 6C, and 45% in Fig. 6D. In figure 6F the effect of SENP3 knockdown is significant at ~19%. Overall, while there is some variation between experiments, we believe this is well within acceptable limits, the results are internally consistent and we have normalized the data in the most appropriate manner.

References

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Uo T, Kinoshita Y, Morrison RS (2005) Neurons exclusively express N-Bak, a BH3 domain-only Bak isoform that promotes neuronal apoptosis. *J Biol Chem* **280**: 9065-9073

Acceptance Letter 27 February 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been re-reviewed by referee 2. As you can see below, the referee appreciates the introduced changes and support publication here. I am therefore very pleased to accept the paper for publication here.

A few minor editorial comments:

1) I noticed that in the figure legends for figure 1 and 5B that the statistical method used and the number of replicates is not specified. Please double-check the other figure legends as well. You can send me by email an amended word file.

2) We also now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

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REFEREE REPORT

Referee #2

The authors have adequately answered to my previous questions.

