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MCL-1 is a stress sensor that regulates autophagy in a developmentally regulated fashion

Marc Germain, Angela P. Nguyen, J. Nicole Le Grand, Nicole Arbour, Jacqueline L. Vanderluit, David S. Park, Joseph T. Opferman and Ruth S. Slack

Corresponding author: Ruth Slack, University of Ottawa

Transaction Report:

(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.)

1st Editorial Decision   21 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as interesting in principle they also think that the conclusiveness and completeness of the experimental evidence provided is not sufficient at this point to justify the conclusions drawn. I will not repeat all their individual points of criticism here, but apart from certain other issues (including limited insight into the mechanism of how Mcl-1 regulates autophagy) the major concern is that the evidence provided is not considered as sufficient to separate the effects of Mcl-1 on apoptosis versus autophagy in vivo in a convincing manner. Clearly, the referees point to major shortcomings of key aspects of the experimental evidence provided, and an extensive amount of further experimentation would be required to address these issues. Furthermore, the outcome of such experiments cannot be predicted at this point. I am afraid that I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point.

We would, however, be able to consider a new submission on this topic should future experiments allow you to strengthen the in vivo aspect of the study considerably and to discriminate between effects of Mcl-1 on apoptosis and autophagy during neuronal differentiation in a considerably more convincing manner. I need to stress, however, that if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh (involving the original referees if available at the time of resubmission), also

with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript by Germain et al describes Mcl-1 as a novel regulator of cell fate during nutrient deprivation, and suggests a role of Mcl-1 in maintaining neuronal survival. This is a very well performed study, however a few issues will need to be addressed.

Major Criticism:

1. My main concern regards to a potential over-interpretation of the in vivo data presented. The authors evaluated Bax activation vs. autophagy markers in brain section of the Mcl-1 deficient mice, and concluded that, based on counting of active Bax/active caspase-3 and punctate LC3-positive cells, autophagy was much more prevalent and hence contributed more significantly to the decrease in cortical tissue mass.

The problem with this comparison is that apoptosis is a very rapid process (occuring within minutes), while autophagy is constitutively active in cells and can be activated for several days in neurons without any resulting damage. It is therefore misleading to compare active Bax-positive with punctate LC3 poisitive cells. To validate the assumption that autophagy activation is more important than apoptosis activation, a genetic approach would be required (e.g. cross-breeding with bax-deficient mice). While these experiments would not be within the scope of this study, I would recommend to very signficiantly tone down the interpretation and discussion of the data.

2. The authors should explain why there is only a 80 % increase in active Bax-positive cells in the Beclin-1 +/- Mcl 1-deficient mice (see comments above).

3. Histology of the Beclin-1 +/- Mcl-1-deficient mice should be provided.

Minor criticism:

4. Scale bars are missing in virtually all immuofluorescence and immunohistochemistry images.

5. Fif 1C: Please check for Bcl-w, the major Bcl-2 family species in the mammalian brain.

6. Fig. 5 D appears to be out of focus. Improved images should be provided.

7. The Discussion is rather short and needs to include mechanisms of Mcl-1 downregulation during nutrient deprivation, and on how autophagy may kill neurons. For example, key work from the Tolkovsky lab should be cited.

Referee #2 (Remarks to the Author):

The paper by Germain et al. shows that the Bcl-2 family protein MCL-1 is rapidly degraded after

aminoacid or glucose/pyruvate starvation in HeLa and H1299 cell lines. This also holds occurs in CGN neurons after hypoxia/reoxygenation. This degradation does not induce cell death but induces Bax activation and Cyt c translocation after aminoacid starvation when the BH3 only protein Bid is over-expressed. MCL-1 degradation occurs after starvation induced autophagy Further MCL-1 overexpression reduces autophagosome number after starvation and its downregulation with siRNA induces autophagosome formation and induces autophagy determined by LC3II lipidation and p62 degradation. Mice with specific deletion of MCL-1 in cortical neurons are born at the expected ratios but manifest reduced size and died before three months of age. These animals showed neuron loss in the corpus callosum at day p14. In addition the mice showed increased autophagy induction as determined by LC3 positive cells identified as neurons and not glial cells, increased number of autophagosomes by EM and p62 degradation by westernblot. MCL-1 deletion also induced Bax and caspase-3 activation. Then the author's study autophagy levels in embryos where MCL-1 is deleted in neural precursors and show that there is no differences in the LC3 staining between wt and MCL-1 deleted cells. Last by comparing neural progenitors and differentiated neurons they conclude that several apoptotic genes are more expressed in neurospheres (BIM, Puma) while some autophagy regulators (Atg9 and Atg7) expression were higher in differentiated neurons. Although both cell types induced autophagy at similar levels after aminoacid starvation, neurospeheres induced caspase-3 activation while neurons did not. Last MCL-1 deleted mice where crossed with Beclin +/- animals manifesting reduced life span and increased Bax and Cyc c expression. In conclusion the author's show that MCL-1 is a sensor for cell stress and may regulate autophagy and apoptosis.

This complex manuscript reaches several interesting conclusions, however many of them that should be further strengthened. Here are my comments:

Major points:

1. Figure 1F and G, why the cells are incubated in the presence of zVAD? This should be justified and mentioned in the text, not only in the figure legend. What are the levels of cell death without caspase inhibition?

2. Figure 2: The author's show that MCL-1 is degraded after glucose/pyruvate starvation and autophagy is activated. Does this occur with similar kinetics after other stimuli that also induce apoptosis and autophagy, such as etoposide or ER-stress, etc...?

3. Figure 2A. What is the incubation time on figure 2A?, in the text it is stated 2h and in the legend 4h. This should be clarified. The levels of MCL-1 should be shown in this figure. Cyt c result from this figure should be mentioned. Is this also performed in the presence of zVAD? If not why?

4. Page 6, sentence: "Under these conditions, loss of MCL-1 protein was an early event, as it occurred before LC3 processing and p62 degradation (Figure 1C)". Do the author's mean Figure 2C? If this is correct, why is so few MCL-1 degradation in this figure in comparison with Figure 1C? (I am aware that the incubation time is 1h instead of 2h as in figure 1C, but the differences are too striking).

5. Page 6 last paragraph, the authors state "The observation that MCL-1 degradation is associated with activation of autophagy, but occurs before major changes in autophagy markers", this can be concluded only the above sentence is relates to Figure 2C and not 1C as it is stated in the text.

6. Figure 5B: the authors conclude that apoptosis induction is very low in the MCL-1 deleted mice however, 30% of cells with active Bax is in my opinion a quite elevated number, thus MCL-1 deletion in mice would induce both autophagy and apoptosis. In addition due to a very efficient clearance of apoptotic cells in tissues, apoptosis could have been underestimated. Thus the author's should provide further evidences that support the conclusion that MCL-1 deletion preferentially induces autophagy and not apoptosis.

7. The author's observe less cells in the corpus callosum after MCL-1 deletion (Figure 4), is this consequence of cell death? Apoptosis should be determined in sections with activated caspase-3 staining.

8. Figure 5C is not described in the text.

9. After figure 5D the author's conclude that autophagy is not activated during embryonic stages E15.5 after deletion of MCL-1 in neural precursors. This conclusion cannot be drawn form the results shown in figure 5D and should be strengthen by determination the same parameters they have used to conclude autophagy induction in figure 5A and B, (i.e. p62 levels, LC3 lipidation, Bax, caspase 3 activation etc...)

10. Figure 6A, Atg5-12 is not shown. Atg9 is not described. From this figure it is concluded that autophagy is low in neurospheres and apoptosis is high. This is an over statement, other markers should be used for both autophagy and apoptosis.

11. From the data presented in figure 6B it is concluded that neurospheres are more susceptible that neurons to nutrient starvation as they induce caspase-3 activation. What are the levels of cell death in both neurons and neurospheres determined y other means besides caspase-3 cleavage in blot?, Is this particular for aminoacid starvation? to further support this conclusion the authors should investigate other cell death stimuli, as glucose/pyruvate, growth factor deprivation, etc...

12. Endogenous Beclin-1 co-precipitated with endogenous MCL-1 in H1299 cells (Figure 6C), suggesting that MCL-1 might regulate autophagy at least in part by controlling the activity of Beclin-1. This has been previously shown (Erlich, Autophagy 2007) the authors should at least comment that previous result.

13. Last the author's should find a suitable neuronal differentiation paradigm (for example in vitro differentiation of neurospheres) to manipulate MCL-1 and determine the autophagy and apoptosis response during stress before and after neuronal differentiation.

Minor points:

1. The author's forgot to mention important evidences form the literature, they should mention and discuss new references in the introduction and discussion, here are some suggestions:

- The reference Germain and Slack is incorrect, it should be year 2009 instead of 2010 (as it appears in Pubmed). In my opinion that reference is not a valid reference regarding the general process of autophagy.

- In the introduction the author's should cite the work of David Rubinsztein and co-workers as the first evidence that induction of autophagy was cytoprotective during neurodegenerative states (Ravikumar, Nature Genetics 2004)

- The author's also forget to mention the first relationship between autophagy and apoptosis (Boya, MCB 2005).

- The author's should mention the relevance of other autophagy proteins such as Ambra during development (Fimia, Nature 2007)

- Beclin has been previously shown to interact with MCl-1 (Erlich, Autophagy 2007).

- MCL-1 regulates autophagy-dependent cell killing in cancer cells (Martin Cancer Biol Ther. 2009)

2. The discussion section should be improved by mentioning for example the result of MCL-1 deletion in other systems.

3. Statistical methods used should be described.

Referee #3 (Remarks to the Author):

This paper describes that MCL-1, a Bcl-2 homolgue, regulates autophagy. The paper tries to make a story about the possibility that MCL-1 is a regulator of decisions of cells to die by apoptosis or induce autophagy.

Unfortunately the paper has two major problems. It does not describe how MCL-1 regulates autophagy. If this is via the same mechanism as Bcl-2, then this would not be very exciting. Also, the inferences of apoptotis versus autophagy are not as clearcut as the authors would propose.

Specific comments:

1. P5 - the authors suggest their results show that a second proapoptotic signals is required to drive the MCL-1 deficient cells into apoptosis. However, there is a lot of apoptosis in the tBID cells either starved or unstarved in 1F and the difference between the starved ands unstarved cells is not significant in 1F. I do agree that loss of MCL-1 does not cause apoptosis by itself, but the data showing that it really potentiates the cell apoptosis are not so strong.

2. It would be good to show the data in 1C in neurons as this is a focus of the study.

3. Fig 2B needs quantification

4. Fig 2D raises issues about using p62 as a readout for autophagy in the contexts of this study as its levels decrease in 3MA treated cells that are starved. The 3MA should block autophagy. This affects many of the other figs using np62, like 3C.

5. Fig 3A, 3G - quantify

6. Fig 4E,F and 5D need higher magnifications - I cannot make out the autophagosomes easily.

7. Fig 4H needs quantification

8. The data comparing autophagy markers with apoptotic markers in the paper in the knockout mice are like comparing apples and pears. The two are not comparable - both are elevated. The apoptosis may be an underestimate as apoptotic cells are rapidly cleared. Also, numbers of LC3 dots can accumulate in the brain due to increased formation but also due to decreased clearance, and these possibilities are not resolved. I appreciate that they are difficult to resolve in the brain, but this is critical for interpretations.

9. The comparisons in the beclin-deficient animals are also difficult to interpret for the following reasons. Autophagy deficiency makes cells more susceptible to apoptotic insults Cell Death Differ. 2010 Feb;17(2):268-77. But, apoptosis also inhibits autophagy (Luo et al Cell Death Differ. 2010 Feb;17(2):268-77). Because of these interdependencies, it is very difficult to interpret the data.

10. Fig 6A shows no data with ATG5-12 conjugate as described in the text on p 10.

11. Fig 6a - The authors need to show changes in autophagosom formation and autophagic flux (delivery of substrates to lysosomes). ATG9 and ATG7 (or ATG5-12) levels are not proper measures of autophagy.

12. P 11 - it is known that inhibition of autophagy makes cells more susceptible to apoptosis (Boya et al Mol Cell Biol. 2005 Feb;25(3):1025-40.)

Resubmission   26 August 2010

Response to reviewers:

Referee #1 (Remarks to the Author):

The manuscript by Germain et al describes Mcl-1 as a novel regulator of cell fate during

5

nutrient deprivation, and suggests a role of Mcl-1 in maintaining neuronal survival. This is a very well performed study, however a few issues will need to be addressed.

Major Criticism:

1. My main concern regards to a potential over-interpretation of the in vivo data presented. The authors evaluated Bax activation vs. autophagy markers in brain section of the Mcl-1 deficient mice, and concluded that, based on counting of active Bax/active caspase-3 and punctate LC3-positive cells, autophagy was much more prevalent and hence contributed more significantly to the decrease in cortical tissue mass.
The problem with this comparison is that apoptosis is a very rapid process (occuring within minutes), while autophagy is constitutively active in cells and can be activated for several days in neurons without any resulting damage. It is therefore misleading to compare active Bax-positive with punctate LC3 poisitive cells. To validate the assumption that autophagy activation is more important than apoptosis activation, a genetic approach would be required (e.g. cross-breeding with bax-deficient mice). While these experiments would not be within the scope of this study, I would recommend to very signficiantly tone down the interpretation and discussion of the data.

We agree with the reviewer that it is difficult to directly compare autophagy and apoptosis in tissue. We have nonetheless addressed the reviewer's concerns by carrying out additional experiments addressing the relationship between autophagy and apoptosis in MCL-1 deficient neurons. First, we addressed the temporal relationship between autophagic and apoptotic markers in the affected cell population of the cortex of MCL-1 KO mice. The new Figure 5G documents that virtually all (97±3%) NeuN-positive neurons are autophagic (LC3-positive) in P7 MCL-1 KO mice. In addition, all BAXpositive cells are also LC3-positive at P7 (New Figure 6C), suggesting that the apoptotic cells are a subset of the autophagic cells. As the number of apoptotic neurons increased over time (Figure 5D) and no new neurons are generated in this region after birth, we concluded that the initial response of neurons to MCL-1 loss is induction of autophagy. In addition, we addressed the concerns over the rapid disappearance of apoptotic cells *in vivo* by performing additional *in vitro* experiments, a setting in which apoptotic cells are not rapidly removed. Here, *in vitro* deletion of MCL-1 did not induce any apoptosis in cortical neurons over wild type levels, even after 10 days (New Figure 6D and E), supporting our interpretation that MCL-1 deficiency induces a robust autophagic response in mature neurons. Our conclusions are also supported by data indicating that neural progenitors are more susceptible to apoptosis than neurons under starvation conditions (Figure 7, including New Figure 7B and E; note the presence of active caspase-3 in E12.5 Foxg1 brains but not P1-14 CamKII brains (Figure 6A)). Taken together, these additional experiments provide further support to our interpretation that loss of MCL-1 in post-mitotic neurons results in an autophagic response. We have nevertheless acknowledged that loss of MCL-1 in the cortex induces apoptosis (p. 10) and toned down our interpretation and discussion of the data.

2. The authors should explain why there is only a 80 % increase in active Bax-positive cells in the Beclin-1 +/- Mcl 1-deficient mice (see comments above).

Deletion of one allele of Beclin-1 only reduces autophagy by 30-50% (Qu et al., 2003 *J. Clin. Invest.*; Yue et al., 2003 *Proc. Natl. Acad. Sci. USA*). It is because of this partial effect on autophagy that we only see a partial effect on BAX activation. This is now clearly stated in the text (p. 12)

3. Histology of the Beclin-1 +/- Mcl-1-deficient mice should be provided.

Histology for the MCL-1 KO Beclin-1 het is now presented in Figure 8A

Minor criticism:

4. Scale bars are missing in virtually all immuofluorescence and immunohistochemistry images.

Scale bars have been added.

5. Fif 1C: Please check for Bcl-w, the major Bcl-2 family species in the mammalian brain.

Figure 1C now shows expression of BCL-2 homologues (including MCL-1 and BCL-W) in starved primary cortical neurons. This new result provides further support for our conclusion that levels of BCL-2 homologues other than MCL-1 are not affected by starvation.

6. Fig. 5 D appears to be out of focus. Improved images should be provided.

Additional higher magnification images are now provided in Figure 7D (originally Figure 5D) to show the presence of autophagosomes.

7. The Discussion is rather short and needs to include mechanisms of Mcl-1 downregulation during nutrient deprivation, and on how autophagy may kill neurons. For example, key work from the Tolkovsky lab should be cited.

The discussion has now been improved using the reviewers' suggestions, including the mechanism of MCL-1 degradation and autophagic cell death in neurons.

Referee #2 (Remarks to the Author):

This complex manuscript reaches several interesting conclusions, however many of them that should be further strengthened. Here are my comments:

Major points:

1. Figure 1F and G, why the cells are incubated in the presence of zVAD? This should be justified and mentioned in the text, not only in the figure legend. What are the levels of cell death without caspase inhibition?

As BH3-only proteins cause BAX activation and cytochrome c release upstream of caspase activation, zVAD was included in the experiment in Figure 1F and G as a way to prevent cells from lifting from the coverslips, as previously described (Germain et al, EMBO J 2005; Germain and Duronio, JBC 2007). In the absence of a caspase inhibitor, the observed cytochrome c release is reduced as some cells detach from the coverslip. This is now explained in the text.

2. Figure 2: The author's show that MCL-1 is degraded after glucose/pyruvate starvation and autophagy is activated. Does this occur with similar kinetics after other stimuli that also induce apoptosis and autophagy, such as etoposide or ER-stress, etc...?

To address this, we conducted similar experiments under other conditions including DNA damage and deprivation of essential amino acids (EBSS). We show that MCL-1 degradation occurred at a similar rate after camptothecin or EBSS treatment as shown in the new Figure 1E. Rapid degradation of MCL-1 following UV and etoposide treatments has also previously been reported (Nijhawan *et al. Genes Dev.* 2003)

3. Figure 2A. What is the incubation time on figure 2A?, in the text it is stated 2h and in the legend 4h. This should be clarified. The levels of MCL-1 should be shown in this figure. Cyt c result from this figure should be mentioned. Is this also performed in the presence of zVAD? If not why?

Cells in Figure 2A and B were incubated for 4h, as stated in the legend. We apologize for the confusion. The text has been corrected and Cyt c results mentioned (p. 6). Figure 2A was also modified to include MCL-1 levels, as requested by the reviewer. Only the experiment described in Figure 1F-G and Figure 7E were carried out in the presence of

zVAD as none of the other experiments caused cells lifting from plate. EBSS treatments gave the same results in the presence or absence of zVAD (not shown).

4. Page 6, sentence: "Under these conditions, loss of MCL-1 protein was an early event, as it occurred before LC3 processing and p62 degradation (Figure 1C)". Do the author's mean Figure 2C? If this is correct, why is so few MCL-1 degradation in this figure in comparison with Figure 1C? (I am aware that the incubation time is 1h instead of 2h as in figure 1C, but the differences are too striking).

We apologize for the mislabeling of the figure. It is indeed Figure 2C and not 1C. As for the difference between 1h and 2h incubation in EBSS, this is likely due to the difference in incubation time as well as the fact that the blot in Figure 1C was exposed for a shorter time that the one in Figure 2C.

5. Page 6 last paragraph, the authors state "The observation that MCL-1 degradation is associated with activation of autophagy, but occurs before major changes in autophagy markers", this can be concluded only the above sentence is relates to Figure 2C and not 1C as it is stated in the text.

As stated in response to the previous comment, the actual figure that is referred to is Figure 2C, we have now corrected the figure labeling.

6. Figure 5B: the authors conclude that apoptosis induction is very low in the MCL-1 deleted mice however, 30% of cells with active Bax is in my opinion a quite elevated number, thus MCL-1 deletion in mice would induce both autophagy and apoptosis. In addition due to a very efficient clearance of apoptotic cells in tissues, apoptosis could have been underestimated. Thus the author's should provide further evidences that support the conclusion that MCL-1 deletion preferentially induces autophagy and not apoptosis.

We agree with the reviewer that the response we observe in MCL-1 KO animals is partly apoptotic and have acknowledged this in the text (p.10). However, we now present new experimental evidence that supports our hypothesis that the initial response in postmitotic neurons is autophagic (note this has also been described in detail in Point 1 for reviewer #1):

We first addressed the temporal relationship between autophagic and apoptotic markers in the affected cell population of the cortex of MCL-1 KO: Virtually all (97±3%) neurons (NeuN-positive cells) are also positive for LC3 in P7 mice (New Figure 5G) while all BAX-positive cells are also LC3-positive at P7 (New Figure 6C), thus suggesting that apoptotic cells are a subset of the autophagic cells. As the number of apoptotic neurons increased over time (Figure 5D) and no new neurons are generated in this region after birth, we concluded that the initial response of neurons to MCL-1 loss is induction of autophagy. In addition, we addressed the concerns over the rapid disappearance of apoptotic cells *in vivo* by performing additional *in vitro* experiments, a setting in which apoptotic cells are not rapidly removed. Here, *in vitro* deletion of MCL-1 did not induce any apoptosis in cortical neurons over wild type levels, even after 10 days (New Figure 6D and E), supporting our interpretation that MCL-1 deficiency induces a robust autophagic response in mature neurons. Our conclusions are also supported by data indicating that neural progenitors are more susceptible to apoptosis than neurons in MCL-1 KO and under starvation conditions (Figure 7, including new experiments suggested by the reviewer and presented in New Figure 7B and E; note the presence of active caspase-3 in E12.5 Foxg1 brains but not P1-14 CamKII brains (Figure 6A)). Taken together, these additional experiments provide further support to our interpretation that loss of MCL-1 in post-mitotic neurons results in an autophagic response. We nevertheless toned down our interpretation and discussion of the data.

7. The author's observe less cells in the corpus callosum after MCL-1 deletion (Figure 4), is this consequence of cell death? Apoptosis should be determined in sections with

activated caspase-3 staining.

As the corpus callosum is mainly constituted of axons, the apparent loss of cells in the corpus callosum is the consequence of axonal degeneration of the neurons in the cortical layers adjacent to it. The text has been modified on p. 9 to better explain this result.

8. Figure 5C is not described in the text.

The Figure was improperly referred to as Figure 4C. This has been corrected.
9. After figure 5D the author's conclude that autophagy is not activated during embryonic stages E15.5 after deletion of MCL-1 in neural precursors. This conclusion cannot be drawn form the results shown in figure 5D and should be strengthen by determination the same parameters they have used to conclude autophagy induction in figure 5A and B, (i.e. p62 levels, LC3 lipidation, Bax, caspase 3 activation etc...)

We now provide additional data in Figure 7B showing activation of caspase-3 in E12.5 Foxg1-MCL-1 KO, but not in P1-14 CamKII MCL-1 KO (consistent with our previous results (Arbour 2008); see Figure 6A for caspase-3 in CamKII mice). In contrast, LC3-II levels were similar between control and MCL-1 KO, suggesting that there is no major difference in autophagy between the two phenotypes.

10. Figure 6A, Atg5-12 is not shown. Atg9 is not described. From this figure it is concluded that autophagy is low in neurospheres and apoptosis is high. This is an over statement, other markers should be used for both autophagy and apoptosis.

This was our mistake. The blot actually shows ATG5 as described in the text. As for different levels of apoptosis and autophagy in neurons and neurospheres, we did not claim that their actual levels change but rather suggested that that their propensity to activate one or the other is different in response to stress (this is now clarified on p.12). We tested this hypothesis in Figure 7D and New Figure 7E. We agree with the reviewer that actually demonstrating a difference in basal levels of autophagy and apoptosis would require additional experiments, which are beyond the scope of this paper.

11. From the data presented in figure 6B it is concluded that neurospheres are more susceptible that neurons to nutrient starvation as they induce caspase-3 activation. What are the levels of cell death in both neurons and neurospheres determined y other means besides caspase-3 cleavage in blot?, Is this particular for aminoacid starvation? to further support this conclusion the authors should investigate other cell death stimuli, as glucose/pyruvate, growth factor deprivation, etc...

We have now added data for cytochrome c release in starved neurons and neurospheres (new Figure 7E) supporting our hypothesis that starved neurospheres are more susceptible to apoptosis than neurons. Data is presented for both EBSS and glucose starvation.

12. Endogenous Beclin-1 co-precipitated with endogenous MCL-1 in H1299 cells (Figure 6C), suggesting that MCL-1 might regulate autophagy at least in part by controlling the activity of Beclin-1. This has been previously shown (Erlich, Autophagy 2007) the authors should at least comment that previous result.

We now cite the reference when presenting data on the interaction between MCL-1 and Beclin-1 (p. 8).

13. Last the author's should find a suitable neuronal differentiation paradigm (for example in vitro differentiation of neurospheres) to manipulate MCL-1 and determine the autophagy and apoptosis response during stress before and after neuronal differentiation.

We are able to induce differentiation of neurospheres. However, the percentage of neurons generated is relatively low <20%, which would make interpretation difficult

particularly for western analysis. Thus, in the new experiments added to address this point, from the brain of 1 embryo (n=1) we removed neural precursors for neurosphere cultures and cortical plate neurons for primary neuronal cultures. These preparations enabled us to compare relatively pure populations of undifferentiated progenitors versus primary cortical neurons.

Minor points:

1. The author's forgot to mention important evidences form the literature, they should mention and discuss new references in the introduction and discussion, here are some suggestions:
- The reference Germain and Slack is incorrect, it should be year 2009 instead of 2010 (as it appears in Pubmed). In my opinion that reference is not a valid reference regarding the general process of autophagy.
- In the introduction the author's should cite the work of David Rubinsztein and coworkers as the first evidence that induction of autophagy was cytoprotective during neurodegenerative states (Ravikumar, Nature Genetics 2004)
- The author's also forget to mention the first relationship between autophagy and apoptosis (Boya, MCB 2005).
- The author's should mention the relevance of other autophagy proteins such as Ambra during development (Fimia, Nature 2007)
- Beclin has been previously shown to interact with MCl-1 (Erlich, Autophagy 2007).
- MCL-1 regulates autophagy-dependent cell killing in cancer cells (Martin Cancer Biol Ther. 2009)

The references suggested by the reviewer are now cited in the introduction and the discussion. The reference Germain and Slack has also been replaced by a more appropriate general autophagy review (Mehrpour *et al* (2010) *Cell Res.* 20 p.748) when discussing general autophagy regulation.

2. The discussion section should be improved by mentioning for example the result of MCL-1 deletion in other systems.

The discussion has been improved reviewers' suggestions, including the mention of MCL-1 deletion in other systems (p. 14)

3. Statistical methods used should be described.

Statistical methods have now been included in the Methods section (p.20)

Referee #3 (Remarks to the Author):

This paper describes that MCL-1, a Bcl-2 homolgue, regulates autophagy. The paper tries to make a story about the possibility that MCL-1 is a regulator of decisions of cells to die by apoptosis or induce autophagy.

Unfortunately the paper has two major problems. It does not describe how MCL-1 regulates autophagy. If this is via the same mechanism as Bcl-2, then this would not be very exciting. Also, the inferences of apoptotis versus autophagy are not as clearcut as the authors would propose.

We now provide more data documenting the interaction between MCL-1 and Beclin-1, which has been shown to prevent Beclin-1 function in overexpression studies (Erlich, Autophagy 2007, Mauri EMBO 2007). Importantly, we now provide evidence that this occurs at the mitochondria and not the ER (New Figure 4), where BCL-2 and BCL-XL functionally interact with Beclin-1 (Mauri EMBO 2007, Pattingre Cell 2005). This is an importance difference, especially in the light of recent findings suggesting a role for mitochondrial membranes in the formation of the autophagosome (Hailey DW Cell 2010). As for the relationship between autophagy and apoptosis, we have performed additional experiments to address this important issue (discussed in detail below).

Specific comments:

1. P5 - the authors suggest their results show that a second proapoptotic signals is required to drive the MCL-1 deficient cells into apoptosis. However, there is a lot of apoptosis in the tBID cells either starved or unstarved in 1F and the difference between the starved ands unstarved cells is not significant in 1F. I do agree that loss of MCL-1 does not cause apoptosis by itself, but the data showing that it really potentiates the cell apoptosis are not so strong.

We agree with the reviewer that the observed difference between nutrient-replete and starved cells is not enormous. It should be pointed out, however, that because of the conditions of the experiment (we are starving cells, thereby inhibiting translation half way through the infection), the amount of tBID expressed in starved cells is about one third of the amount in nutrient-replete cells. Therefore, the amount of BAX activation and cytochrome c release in starved cells is likely underestimated. Nevertheless, the experiment was repeated to determine whether BAX activation is actually significantly increased in starved cells treated with Ad tBID. This new data indicates that both BAX activation and cytochrome c release are significantly increased in starved cells expressing tBID compared to tBID alone.

2. It would be good to show the data in 1C in neurons as this is a focus of the study.

This is now shown in Figure 1C

3. Fig 2B needs quantification

The surface area covered by LC3-positive vesicles under each condition is now shown under Figure 2B.

4. Fig 2D raises issues about using p62 as a readout for autophagy in the contexts of this study as its levels decrease in 3MA treated cells that are starved. The 3MA should block autophagy. This affects many of the other figs using np62, like 3C.

The reviewer raises a valid point concerning the use of p62 as an autophagy marker. To confirm that p62 levels are indeed regulated by lysosomal degradation following induction of autophagy, we carried additional experiments in which lysosomal degradation was prevented by bafilomycin. As shown in the new Figure 2E, this treatment completely prevented p62 degradation but had no effect on MCL-1 degradation. Therefore, we think that the partial effect of 3MA on p62 levels reflects issues with 3MA efficiency rather than p62 as an autophagy marker. In any case, we measured LC3, along with p62, in all subsequent experiments to alleviate possible issues with each autophagy marker.

5. Fig 3A, 3G – quantify

Figure 3A was quantified in B. Quantification for Figure 3G (now Figure 3H) is quantified in New Figure 3G .

6. Fig 4E,F and 5D need higher magnifications - I cannot make out the autophagosomes easily.

Magnified images were added for figure 4F and 5D (now Figures 5F and 7A). Please see Figure 5F for a magnification of Figure 5E. Figure 5E is shown only to document the region of the cortex that is affected.

7. Fig 4H needs quantification

The surface area occupied by autophagosomal structures in control and MCL-1 KO has been quantified and is now shown in New figure 5I.

8. The data comparing autophagy markers with apoptotic markers in the paper in the knockout mice are like comparing apples and pears. The two are not comparable - both are elevated. The apoptosis may be an underestimate as apoptotic cells are rapidly cleared. Also, numbers of LC3 dots can accumulate in the brain due to increased formation but also due to decreased clearance, and these possibilities are not resolved. I appreciate that they are difficult to resolve in the brain, but this is critical for interpretations.

We agree with the reviewer that it is difficult to directly compare autophagy and apoptosis, especially since apoptotic cells rapidly disappear from the tissue. However, we now present new experimental evidence that supports our hypothesis that the initial response in post mitotic neurons is autophagic (note this has also been described in detail in Point 1 for reviewer #1):

The temporal relationship between autophagic and apoptotic markers in the affected cell population of the cortex of MCL-1 KO mice suggests that affected neurons first show signs of autophagy, then apoptosis: Virtually all neurons (NeuN-positive cells) are also positive for LC3 in P7 mice (New Figure 5G) while all BAX-positive cells are also LC3-positive at P7 (New Figure 6C), thus suggesting that apoptotic cells are a subset of the autophagic cells. As the number of apoptotic neurons increased over time (Figure 5D) and no new neurons are generated in this region after birth, we concluded that the initial response of neurons to MCL-1 loss is induction of autophagy. In addition, we addressed the concerns over the rapid disappearance of apoptotic cells *in vivo* by performing additional *in vitro* experiments, a setting in which apoptotic cells are not rapidly removed. Here, *in vitro* deletion of MCL-1 did not induce any apoptosis in cortical neurons over wild type levels, even after 10 days (New Figure 6D and E), supporting our interpretation that MCL-1 deficiency induces a robust autophagic response in mature neurons. Our conclusions are also supported by data indicating that neural progenitors are more susceptible to apoptosis than neurons in MCL-1 KO and under starvation conditions (Figure 7, including New Figure 7B and E; note the presence of active caspase-3 in E12.5 Foxg1 brains but not P1-14 CamKII brains (Figure 6A)). Taken together, these additional experiments provide further support to our interpretation that loss of MCL-1 in postmitotic neurons results in an autophagic response. We nevertheless toned down our interpretation and discussion of the data.

Concerning the LC3 staining, as the reviewer points out, the determination of autophagic flux in tissue is challenging. To address this question, we measured the levels of p62 (degraded in an autophagy-dependent manner) as a measure of lysosomal degradation of autophagosomes. The decrease in p62 we observed in MCL-1 KO mice (Figure 5A) is consistent with an effect of MCL-1 on the initiation of autophagosome formation rather than fusion with lysosomes, as data in cell lines suggested (Figure 1-3). This has been clarified in the text (p. 9)

9. The comparisons in the beclin-deficient animals are also difficult to interpret for the following reasons. Autophagy deficiency makes cells more susceptible to apoptotic insults Cell Death Differ. 2010 Feb;17(2):268-77. But, apoptosis also inhibits autophagy (Luo et al Cell Death Differ. 2010 Feb;17(2):268-77). Because of these interdependencies, it is very difficult to interpret the data.

Our *in vivo* data indeed supports the idea, first described in stressed cell lines, that inhibition of autophagy promotes apoptosis, *(Boya et al Mol Cell Biol. 2005 Feb;25(3):1025-40.)*. In that respect, our observation that MCL-1 can regulate autophagy in addition to its well-characterized anti-apoptotic function provides some insights as to how the two processes could be related. Our new data showing an increase in 6A7-positive/LC3-negative in P14 MCL-1 KO Beclin-1+/- animals (New Figure 8D) also provides *in vivo* support for the hypothesis that activated caspases can turn off the autophagic process (Luo et al Cell Death Differ. 2010 Feb;17(2):268-77).

10. Fig 6A shows no data with ATG5-12 conjugate as described in the text on p 10.

The blot shown in Figure 6A (Now Figure 7C) was unfortunately mislabeled and is actually ATG5, as stated in the text, not ATG9. We apologize for this confusion.

11. Fig 6a - The authors need to show changes in autophagosom formation and autophagic flux (delivery of substrates to lysosomes). ATG9 and ATG7 (or ATG5-12) levels are not proper measures of autophagy.

We totally agree with the reviewer that if we were claiming that there is an actual change in autophagic flux between neural progenitors and post-mitotic neurons, we would need to carry the experiments suggested. However, in Figure 6A (Now Figure 7C), we are not claiming that there is more autophagy in neurons than neurospheres but rather suggest that neurospheres (neural progenitor cells) are likely more susceptible to activate apoptosis than neurons following autophagy-related stress, as they have higher levels of pro-apoptotic proteins and lower levels of autophagy-related proteins than neurons. We test this hypothesis in Figure 7D and New Figure 7E. We agree with the reviewer that actually demonstrating a difference in basal levels of autophagy and apoptosis would require additional experiments, which are beyond the scope of this paper. This is now clarified on p. 12

12. P 11 - it is known that inhibition of autophagy makes cells more susceptible to apoptosis (Boya et al Mol Cell Biol. 2005 Feb;25(3):1025-40.)

Boya *et al.* and Luo *et al.* are now cited on p. 13. We would like to point out, however, that these experiments were carried in cells that were stressed to elicit an autophagic/apoptotic response. In the absence of stress, inhibition of autophagy did not cause cell death. It is therefore noteworthy that the only manipulation done to the cortical neurons of our study was the deletion of MCL-1 in the animals, providing for the first time *in vivo* evidence of an interplay between the two processes. This is now considered in the discussion p.14

2nd Editorial Decision   21 September 2010

Thank you for sending us a new version of your original manuscript EMBOJ-2010-74597 as a new submission. Our original referees have now seen it again. In general, the referees are now more positive about publication of your paper. However, referees 1 and 3 still think that there are a few issues that need to be addressed (see below) before the data are strong enough to fully justify the conclusions drawn. As this is a resubmission rather than a simple revision there is still the option for one round of revision. I would therefore like to ask you to address the issues raised regarding the conclusiveness of the data by further experimentation. New experiments to address the mechanistic issue raised by referee 1 (point #1) will not be required.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This revision has dealt with many of the queries raised by the reviewers. There are still a few outstanding questions:

1. There is no real mechanism provided for the effects of MCL-1. Simply showing colocalisation in mitochondrial fractions is not enough.

2. I would be happier if the authors did autophagy flux experiments with lysosoaml inhibitors where possible - e.g. Fig 2 and Fig 3. These are actually critical to make the convincing case that MCL-1 regaulates autophagosome formation. As the data stand, this is not so secure (as everything hinges on the p62 data).

3. Fig 3B is a little worrying as it stands, since it suggests that MCL-1 is not required for starvation-induced autophagy. Furthermore, overexpressing or knocking down MCL-1 do not significnatly influence autophagosome vesicel number. This seems to contradict the central message of the paper. This issue may be able to be cleared up by doing further experiments with lysosomal inhibitors. Fig 3H is also worrying, since one would expect that increased autophagic flux would correlate with increased delivery of autophagosome to lysosomes. This does not fit with the data - although again there may be technical issues.

4. Fig 7A - the inferences of autophagic rates changing as neurons differentiate again is confounded by the flux issues. One simply cannot make deductions based on "steady-state" LC3 staining. An increase occurs if there is more formation relative to degradation, but one could actually have increased flux with lower levels of LC3 if there is both more formation of autophagosomes as well as increased degradation. This issue needs to be addressed explicitly and the data downplayed.

Referee #2 (Remarks to the Author):

The authors have adequately revised the manuscript. This is an excellent study.

Referee #3 (Remarks to the Author):

I appreciate the effort the author's have done to improve the manuscript, there are however some issues that still need to be addressed:

- The author's claim that autophagy and not apoptosis is the response after MCL-1 modulation (Figure 3) since no cyt c translocation is observed. They should use a positive control for Cytc translocation and other methods to measure cell death such determining chromatin condensation with DAPI, Bax quantification, Caspase-3 activation etc... as it is done for other figures in the manuscript.
- Author's show increase in autophagosomes after MCL-1 deletion with no signs of cell death after 3h of starvation. Do these cells die at all after starvation?, 3h hours is often not sufficient to induce cell death, longer incubation times should be compared.
- Does MCL-1 regulate autophagy after other stimuli such as rapamycin, oxidative stress or DNA-damage?
- In figure 7B the author's conclude that autophagy is not activated since there are no modifications in LC3II levels. This result should be strengthened with other markers such as p62 (as it is performed in the rest of the manuscript).
- Is MCL-1 degraded after starvation in neurospheres? Is MCL-1 regulating autophagy in these cells? This evidence will strengthen the point that the effects are developmentally regulated.

Other minor points:

- MCL-1 degradation in cortical neurons is rather low (fig 1C). Why there are two bands, which is the specific? The decrease is only observed in one of them. This should be mentioned in the text.
- Several references are in wrong format (Mehrpour et al. Chang et al).

1st Revision - authors' response   12 October 2010

Referee #1 (Remarks to the Author):This revision has dealt with many of the queries raised by the reviewers. There are still a few outstanding questions:

1. There is no real mechanism provided for the effects of MCL-1. Simply showing colocalisation in mitochondrial fractions is not enough.

We totally agree with the reviewer that simply showing colocalisation in mitochondrial fractions is not enough to demonstrate how MCL-1 regulates autophagy. This is not, however, the goal of this paper, which shows that stress-dependent degradation of MCL-1 can lead to apoptosis or autophagy, a response that is context-dependent. We think that such a detailed mechanistic analysis would only dilute the message of the manuscript. In addition, it has already been suggested that MCL-1 can regulate autophagy through its interaction with Beclin-1 (Maiuri EMBO J. 2007). We provided the mitochondrial data only to indicate that anti-apoptotic BCL-2 homologues are distinct with respect to their role in regulation of autophagy (BCL-2 regulates autophagy at the endoplasmic reticulum).

2. I would be happier if the authors did autophagy flux experiments with lysosoaml inhibitors where possible - e.g. Fig 2 and Fig 3. These are actually critical to make the convincing case that MCL-1 regaulates autophagosome formation. As the data stand, this is not so secure (as everything hinges on the p62 data).

As suggested by the reviewer, we now provide additional data supporting our hypothesis that MCL-1 regulates autophagy upstream of autophagosome-lysosome fusion. First, we now show that downregulation of MCL-1 results in the accumulation of cleaved GFP under both basal and starvation conditions (Figure 3D). As this cleaved GFP is generated from GFP-LC3 following fusion of the autophagosome with a lysosome (Klionsky *et al* (2008) *Autophagy*), this indicates that downregulation of MCL-1 does not inhibit the autophagic flux. In addition, we performed the experiment suggested by the reviewer, and found that inhibition of lysosomal proteases with E64 + pepstatin A resulted in an increase in p62 levels in MCL-1 knockdown cells (new Figure 3H). Altogether, the p62 data (Figure 3C-D), the lysosomal cleavage of GFP-LC3 (now in Figure 3D), the experiment with lysosomal inhibitors (new Figure 3H), as well as the colocalisation between LAMP1 and GFP-LC3 (Figure 3G and I; see next point) all support the idea that MCL-1 regulates autophagy upstream of autophagosome-lysosome fusion.

3. Fig 3B is a little worrying as it stands, since it suggests that MCL-1 is not required for starvation-induced autophagy. Furthermore, overexpressing or knocking down MCL-1 do not significnatly influence autophagosome vesicel number. This seems to contradict the central message of the paper. This issue may be able to be cleared up by doing further experiments with lysosomal inhibitors. Fig 3H is also worrying, since one would expect that increased autophagic flux would correlate with increased delivery of autophagosome to lysosomes. This does not fit with the data - although again there may be technical issues.

We are not sure what the reviewer means by this comment as we show here that the degradation of MCL-1 is required for starvation-induced autophagy to proceed. If the reviewer refers to the observation that there is still some induction of autophagy in Ad MCL-1 cells treated with EBSS, this is likely to reflect the fact that MCL-1 is not a core

autophagy protein. Indeed, in similar experiments, overexpression of wt or ER-targeted BCL-2 did not completely prevent autophagosome formation (Pattingre *et al* (2005) Cell).

As for the effect of altered MCL-1 levels, there is a significant change following EBSS treatment for both overexpression and downregulation of MCL-1. If the reviewer refers to the basal levels, the differences are indeed small. However, we do not necessarily expect the low basal autophagosomal numbers to vary. Indeed, overexpression of BCL-2 did not affect nutrient-replete levels of BCL-2 in most cell types (Pattingre *et al* (2005) Cell). In Figure 3G (which is the quantification of Figure 3I (former Figure 3H)), the percentage of the total pool of LC3 vesicles that colocalise with the lysosomal marker LAMP1 is reported. Although we agree with the reviewer that while the total number of LAMP1-positive LC3 vesicles should change when varying MCL-1 levels, the ratio is expected to stay the same (which is what we observe in Figure 3G), again supporting the idea that MCL-1 does not affect fusion between autophagosomes and lysosomes. This is now clarified in Figure 3G and in the text (p. 8). The arrowheads in figure 3I (former Figure 3H) represent examples of colocalisation and do not point to all LAMP1-positive vesicles in each image. Furthermore, our hypothesis that MCL-1 does not affect the delivery of autophagosomes to lysosomes is also supported by the p62 data (Figure 3CD), the new GFP-LC3 data in Figure 3D and the new experiment with lysosomal inhibitors shown in new Figure 3H.

4. Fig 7A - the inferences of autophagic rates changing as neurons differentiate again is confounded by the flux issues. One simply cannot make deductions based on "steadystate" LC3 staining. An increase occurs if there is more formation relative to degradation, but one could actually have increased flux with lower levels of LC3 if there is both more formation of autophagosomes as well as increased degradation. This issue needs to be addressed explicitly and the data downplayed.

We agree with the reviewer that an increase in LC3 could potentially be the consequence of an increased generation of LC3 vesicles or a decrease in their clearance. As measuring autophagic flux *in vivo* is very challenging, we addressed this issue by comparing the percentage of LC3 vesicles that colocalised with the lysosomal marker LAMP1 in the cortex and ventricular zone of E15.5 wild-type embryos, as this method can provide an idea of the autophagic flux (Klionsky *et al*. (2008) Autophagy 4, p.151). A similar percentage of LC3 vesicles colocalised with LAMP1 in each zone (new Figure 7C), suggesting that there was no major differences in the rate at which autophagosomes fuse with lysosomes. This therefore supports the idea that there is potentially more autophagosome formation in the cortex. We nevertheless downplayed the results and suggested that this could potentially be the result of an upregulation of the autophagic machinery (p. 12), which is what was analyzed next, and not necessarily an absolute increase in autophagy.

Referee #2 (Remarks to the Author): The authors have adequately revised the manuscript. This is an excellent study.

Referee #3 (Remarks to the Author): I appreciate the effort the author's have done to improve the manuscript, there are however some issues that still need to be addressed:

- The author's claim that autophagy and not apoptosis is the response after MCL-1 modulation (Figure 3) since no cyt c translocation is observed. They should use a positive control for Cytc translocation and other methods to measure cell death such determining chromatin condensation with DAPI, Bax quantification, Caspase-3 activation etc... as it is done for other figures in the manuscript.

We have now quantified BAX activation following EBSS treatment, in addition to cytochrome c release, and used Ad tBID as a positive control for these experiments (New Figure 2A). (see also next point)

-Author's show increase in autophagosomes after MCL-1 deletion with no signs of cell

death after 3h of starvation. Do these cells die at all after starvation?, 3h hours is often not sufficient to induce cell death, longer incubation times should be compared.

We now show cyt c and BAX data for an extended time course of EBSS treatment in new Figure 2A, along with Ad tBID as a positive control. In control cells, we did not observe signs of apoptosis for up to 12 hours in EBSS. When MCL-1 is acutely downregulated prior starvation, there is a small increase in apoptosis after 8 hours (less than 10% of the cells) but not before. This therefore occurs long after MCL-1 levels are decreased and signs of autophagy are observed (which is within 2 hours of EBSS treatment).

- Does MCL-1 regulate autophagy after other stimuli such as rapamycin, oxidative stress or DNA-damage?

MCL-1 regulates all forms of starvation-induced autophagy we have tested. As for inducers that can also potently activate an apoptotic response (such as DNA damage), the increased sensitivity of MCL-1 null cells to apoptotic cell death precludes the interpretation of the data with respect to autophagy. Also, we did not see a major effect of MCL-1 on rapamycin-induced autophagy. However, we believe this is a complex issue, as different forms of autophagy may be regulated by distinct mechanisms, and we wish to examine this in more detail in a future study.

- In figure 7B the author's conclude that autophagy is not activated since there are no modifications in LC3II levels. This result should be strengthened with other markers such as p62 (as it is performed in the rest of the manuscript).

p62 levels are now shown in Figure 7B. This is also stated in the text p. 12

- Is MCL-1 degraded after starvation in neurospheres? Is MCL-1 regulating autophagy in these cells? This evidence will strengthen the point that the effects are developmentally regulated.

The regulation of autophagy by MCL-1 in neurospheres is indeed an important question. As such, we now provide evidence that MCL-1 is degraded after starvation in neurospheres (new Figure 7E). It is however difficult to interpret this result, as MCL-1 is degraded following induction of both apoptosis and autophagy and we have shown that starvation induces apoptosis in neurospheres (Figure 7F-G). In addition, deletion of MCL-1 in neural precursors (Arbour *et al* J Neurosci 2008) activates apoptosis, precluding any meaningful assessment of the regulation of autophagy in that context. To address this point, the induction of apoptosis following MCL-1 deletion in neural precursors is now referenced in the text (p. 11)

Other minor points:

- MCL-1 degradation in cortical neurons is rather low (fig 1C). Why there are two bands, which is the specific? The decrease is only observed in one of them. This should be mentioned in the text.

The bands represent two isoforms of MCL-1, as both are lost in the MCL-1 KO (Opferman *et al*, 2003; 2005; Figure 5-6). It is unknown whether they represent a phosphorylated form, a cleavage fragment or another modification of the protein. In any case, the isoform specificity is now properly mentioned in the text (p. 5)

- Several references are in wrong format (Mehrpour et al. Chang et al).

References have been corrected

3rd Editorial Decision   22 October 2010

Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again. As you will see below referee 1 still raises concerns regarding the autophagic flux experiments in figure 3 and thinks that the read-out should be (endogenous) LC3-II. In the meantime I have consulted with referee 3, who is more positive overall, on his/her concerns (point 2 and 3). He/she agrees with referee 1 that such data are required. I would therefore like to ask you to address these concerns by further experimentation. Furthermore, I would like to clarify and confirm that further mechanistic work will not be required.

Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

I am still not really convinced by the revisions. The authors do not appear to have grasped some of the key autophagy issues with their data; also some of the new data are not consistent with their model.

1. The mechanism issue. Given that MCL-1 is a Bcl-2 homologue, its effects on autophagy are not really surprising. Further mechanistic work would help us understand if this acted via the same mechanism as Bcl2 and Bcl-xl.

2. Lysosomal inhibitors. The new experiment have not included what I wanted: LC3-II blots +/- inhibitors, so we can see what is going on with LC3-II flux. The new GFP cleavage data in Fig 3D actually do not fit what they show in 3B. In 3B they show that MCL-1 siRNA increases autophagosome number dramatically in starved cells. However, in 3D, the cleaved GFP levels are the same in MCL-1 and control siRNA cells when they are starved.

I am not a great fan of p62 as an autophagy readout as it can be regulated quite strongly at the transcriptional level. The new p62 data in 3H are again problematic. First the levels should increase a lot in wild-type cells treated with the lysosomal inhibitors - this is what most people see but this is not apparent in the fig. Furthermore, this fig suggests that the levels of p62 MCL-1 knockdown results in decreased levels of p62 even in the presence of lysosomal inhibitors - this would argue that at least of some of the effects on p62 are autophagy-independent. Thus, these p62 experiments are not really convincing as the controls do not behave (control knockdown plus lysosomal inhibitors have no effect), and the data suggest that a major effect of the MCL-1 knockdown on p62 is lysosome-independent.

3. The problem with 3B is first no obvious change in vesicle with MCL-1 knockdown or overexpression normal media - this could have been dissected more sensitively using lysosomal inhibitors as suggested previously. What worries me more in a sense is the dramatic effect of the starvation in the MCL-1 knockdown cells - this almost suggests that the effects of the EBSS are MCL-1 independent. Again more sophisticated assays of flux may help to clarify what is really going on.

2nd Revision - authors' response   11 November 2010

Referee #1 (Remarks to the Author):
I am still not really convinced by the revisions. The authors do not appear to have
grasped some of the key autophagy issues with their data; also some of the new data are
not consistent with their model.

1. The mechanism issue. Given that MCL-1 is a Bcl-2 homologue, its effects on
autophagy are not really surprising. Further mechanistic work would help us understand
if this acted via the same mechanism as Bcl2 and Bcl-xl.

The editor did not require us to address this issue. However, we agree with the reviewer
that it is an important question to be addressed in future studies.

2. Lysosomal inhibitors. The new experiment have not included what I wanted: LC3-II
blots +/- inhibitors, so we can see what is going on with LC3-II flux. The new GFP
cleavage data in Fig 3D actually do not fit what they show in 3B. In 3B they show that
MCL-1 siRNA increases autophagosome number dramatically in starved cells. However,
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First the levels should increase a lot in wild-type cells treated with the lysosomal
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of the effects on p62 are autophagy-independent. Thus, these p62 experiments are not
really convincing as the controls do not behave (control knockdown plus lysosomal
inhibitors have no effect), and the data suggest that a major effect of the MCL-1
knockdown on p62 is lysosome-independent.

Figure 3H showing the p62 data has been removed, as we agree with the reviewer that we
cannot draw any conclusion from this experiment. This technical issue has now been
solved by inhibiting lysosomal function with a one-hour bafilomycin treatment
(inhibiting lysosomal acidification; Klionsky *et al*, *Autophagy* 2008) instead of the
protease inhibitors previously used, and blotting for LC3 instead of p62 as requested by
the reviewer. This new data (new Figure 3C-D) demonstrates an increase in endogenous
LC3-II following lysosomal inhibition for both the control siRNA and the MCL-1
siRNA, further supporting our hypothesis that MCL-1 regulates autophagy upstream of
the autophagosome-lysosome fusion step.

As for Figure 3D (Now Figure 3F), we are aware that the amount of free GFP observed
under starved conditions is not proportional to the number of LC3 vesicles in Figure 3B,
assuming that this degradation fragment accumulates linearly with the extent of
autophagy. As cleaved GFP is relatively stable within lysosomes (Klionsky *et al*,
Autophagy 2008), it is however possible that the relationship between autophagosome
numbers and cleaved GFP is not linear when the system is fully activated with EBSS. In
fact, we see an increase in GLP-LC3-II in MCL-1 knockdown cells (Figure 3F) that
correlates with the data in Figure 3B and the new endogenous LC3 data. Therefore, the
only definitive conclusion that can be made from the cleaved GFP data is that GFL-LC3
can be degraded in the lysosomes even when MCL-1 is knocked down, again consistent
with the LC3 and p62 data.

3. The problem with 3B is first no obvious change in vesicle with MCL-1 knockdown or
overexpression normal media - this could have been dissected more sensitively using
lysosomal inhibitors as suggested previously. What worries me more in a sense is the
dramatic effect of the starvation in the MCL-1 knockdown cells - this almost suggests that
the effects of the EBSS are MCL-1 independent. Again more sophisticated assays of flux
may help to clarify what is really going on.

We do understand that a greater increase in autophagosome numbers might be expected when removing a negative regulator of autophagy such as MCL-1, although this is assuming that loss of a negative autophagy regulator would necessarily result in a dominant activation of autophagy. However, another possibility (at least in the case of BCL-2 homologues) is that a stimulus is still required to activate Ulk1 (ATG1) upstream of Beclin-1 and activate autophagy. In that context, loss of MCL-1 would only enhance the effect proportionally to the strength of the upstream stimulus. This hypothesis is supported by our data, as well as from experiments analyzing the role of BCL-2 and BCL-X in autophagy:

1) Knockdown of BCL-2 or the use of the BCL-2 inhibitor ABT737 does not greatly activate autophagy in the absence of a stimulus, but leads to a sensitization to autophagy (Pattingre Cell 2005, Mauiri EMBO J 2007).

2) In the absence of starvation, there is in fact a 1.9±0.3 fold decrease in autophagosome numbers with Ad MCL-1 and a 1.7±0.2 fold increase following knockdown of MCL-1. This correlates with the two-fold reduction in p62 (Figure 3D) and 1.8±0.2 fold increase in LC3-II (Figure 3C) in the MCL-1 knockdown. This is again consistent with the notion that MCL-1 regulates autophagy upstream of lysosomal fusion.

In that context, the effects of EBSS reported in Figure 3B are consistent with a role for MCL-1 in regulating autophagy, as suggested by the rest of our data.