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The IKK complex contributes to the induction of autophagy

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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 16 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I had some difficulty, due to the summer holiday and meeting season, in finding three appropriate reviewers. However, I do now have two reports, and since they are in fair agreement, I feel we can make a decision - to invite a revision - based on these two reports. As you will see, referee 2 is very positive and has only relatively minor comments. Referee 1, while expressing significant interest in your finding that IKK promotes autophagy, raises a number of questions as to the underlying mechanism. Despite the brevity of his/her report, this referee highlights an important concern: you demonstrate that the induction of autophagy is NFkappaB independent, but it remains unclear how IKK is acting in this context. Further mechanistic insight would clearly be very valuable here. In particular, I would highlight the question as to how direct the action of IKK might be.

Given these positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of both referees. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Criollo et al. show that IKK activation can trigger autophagy and that IKK activity is required for induction of autophagy in response to starvation of mTOR inhibition with rapamycin. These are very interesting results that are of potential importance. However, the mechanistic connection between IKK and autophagy is far from clear and as a result, some of the findings appear somewhat paradoxical.

- The authors show that over-expression of activated IKK subunits can induce autophagy. Yet, TNF, a potent IKK activator, does not. What's the explanation?
- Can the induction of autophagy by IKK be indirect, being the consequence of organelle damage or formation of protein aggregates?
- Are AMPK or p53 direct targets for IKK?
- The ability of IKK to increase JNK activity is odd, as IKK usually attenuates JNK activation. In this regard, SP6000125 is quite a non-specific kinase inhibitor.

Referee #2 (Remarks to the Author):

Kroemer and colleagues report that IKK promotes autophagy. Preventing downstream NF-kB activation did not alter autophagy induction by IKK demonstrating that signaling of autophagy by IKK is independent. Importantly, liver-specific knockout of IKK-beta suppressed starvation- or rapamycin-induced autophagy in vivo. Thus, this is an important finding and novel activity of IKK linking it to autophagy regulation. The experiments are well done and there will be substantial interest in this work.

Specific comments:
1. On page 6, in discussion of Hsp90 inhibitors, is it possible that there is an effect on chaperon-mediated autophagy? If so, this possibility should be mentioned.
2. On page 10 the functional assays in supplemental Fig. 7 B and C should not be supplemental.
3. On page 10, what is the functional consequence to starvation or rapamycin treatment to the IKK-beta knockout livers that don't induce efficient autophagy?

1st Revision - Authors' Response 03 November 2009

Referee n° 1 said:

Criollo et al. show that IKK activation can trigger autophagy and that IKK activity is required for induction of autophagy in response to starvation of mTOR inhibition with rapamycin. These are very interesting results that are of potential importance. However, the mechanistic connection between IKK and autophagy is far from clear and as a result, some of the findings appear somewhat paradoxical.

Then pointed out the following major issues:

1. *The authors show that over-expression of activated IKK subunits can induce autophagy. Yet, TNF, a potent IKK activator, does not. What's the explanation?*

Our response: The reviewer wonders why the expression of activated IKK subunits (in particular a constitutively active IKKβ subunit and a plasma-membrane targeted IKKγ/NEMO subunit) can induce autophagy, while TNFα (a potent IKK activator that we employ as a positive control for IKK/NF-κB activation) does not. Most likely, this reflects the complexity of the signal transduction cascades that emanate from the TNF receptor. Indeed, in contrast to the expression of constitutively active IKK subunits (which results in the specific activation of the IKK complex), TNFα induces a panoply of distinct signal transduction events, among which IKK activation is just one. TNFR binding by TNFα causes the assembly of a large supramolecular complex that results from the recruitment at the cytoplasmic side of the plasma membrane of multiple proteins including TRADD, FADD, caspase-9, RIP kinases and TRAFs. This implies that TNFα does not only stimulate IKK activation but rather causes multiple additional signal transduction pathways that are only partially elucidated. (Some of) these molecular cascade must somehow interfere with autophagy induction, and we have discussed this possibility in the paper. We believe that an exhaustive characterization of the TNFα-elicited signal transduction pathways is beyond the scope of this paper. Our article provides molecular genetic evidence that IKK activation suffices to induce autophagy and, more importantly, that IKK inhibition (either by pharmacological means of by knockdown/knockout of IKK subunits) inhibits autophagy induction by physiological and non-physiological stimuli, *in vitro* and *in vivo*.

2. *Can the induction of autophagy by IKK be indirect, being the consequence of organelle damage or formation of protein aggregates?*

   Our response: The referee wonders whether IKK might induce autophagy indirectly by organelle damage or protein aggregation. To address this question, we monitored the subcellular localization of the constitutively active IKKβ subunit (CA-IKKβ) and the plasma membrane-targeted IKKγ/NEMO subunit (MN-NEMO) that were introduced into cells by transfection. As shown in a new supplemental figure (Suppl. Fig. 6), CA-IKKβ and MN-NEMO did not colocalize with GFP-LC3-positive autophagosomes nor with p62 (also known as SQSTM1, a factor that often interacts with protein aggregates). Therefore, we think that protein aggregates formed by IKKβ or IKKγ/NEMO are very unlikely to stimulate autophagy in a non-physiological fashion. Moreover, the chemical inhibition of IKK as well as the siRNA-mediated knockdown or the knockout (by homologous recombination) of each IKK subunit could inhibit autophagy induced by either starvation (which is the physiological inducer of autophagy) or rapamycin (which is the best-characterized pharmacological inducer of autophagy). Altogether, these observations suggest that the induction of autophagy by IKK is not an indirect consequence of organelle damage and/or formation of protein aggregates.

3. *Are AMPK or p53 direct targets for IKK?*

   Our response: While according to Herrero-Martin *et al.* (Embo J, 2009. 28:677-85) AMPK might be a direct substrate direct of TAK1 (which operates upstream of IKK), we were unable to find indications that AMPK might also be a direct target of IKK. It is now well established that p53 levels can be modulated by IKK, through either direct and indirect mechanisms. In 2002, Tergaonkar *et al.* (Cancer Cell, 2002. 1:493-503) identified an IKK-dependent, NF-κB-mediated signaling pathway leading to MDM2-mediatied p53 degradation in response to chemotherapeutic agents (doxorubicin). Moreover, Xia *et al.* (Proc Natl Acad Sci U S A, 2009. 106:2629-34) have recently demonstrated that IKKβ is able to phosphorylate p53 at Ser 362 and 366, thereby promoting its degradation. These observations are in accord with the results presented in this article (Fig. 4C) and provide some further insights into the molecular mechanisms of IKK-induced autophagy. In the revised version of the manuscript, these reports have been cited and briefly discussed in the "Concluding remarks" sections.

4. *The ability of IKK to increase JNK activity is odd, as IKK usually attenuates JNK activation. In this regard, SP600125 is quite a non-specific kinase inhibitor.*

   Our response: Driven by the reviewer's critique we have repeated the experiments involving SP600125 by using an alternative inhibitor of JNK (i.e., the cell permeant peptide H-GRKKRRQRRRPPRPKRPTTLNLFPQVPRSQDT-NH_2, also known as JNK inhibitor I), two different siRNAs that specifically knock down JNK1, as well as siRNAs targeting the obligate JNK1 activators MKK4 and MKK7. Our results demonstrate that the pharmacological or genetic inhibition

of JNK1 (as well as the knockdown of either its upstream activators MKK4 and MKK7) is sufficient to abolish autophagy induction by constitutively active IKK subunits. These results have been incorporated in the revised version of the manuscript within Fig. 4H.

Referee n° 2 said:

Kroemer and colleagues report that IKK promotes autophagy. Preventing downstream NF-κB activation did not alter autophagy induction by IKK demonstrating that signaling of autophagy by IKK is independent. Importantly, liver-specific knockout of IKK-β suppressed starvation- or rapamycin-induced autophagy in vivo. Thus, this is an important finding and novel activity of IKK linking it to autophagy regulation. The experiments are well done and there will be substantial interest in this work.

Then commented:

1. *On page 6, in discussion of Hsp90 inhibitors, is it possible that there is an effect on chaperon-mediated autophagy? If so, this possibility should be mentioned.*

   Our response: The reviewer is correct in pointing out that HSP inhibitors might interfere with chaperone-mediate autophagy. However, our screening system was based on the detection of GFP-$LC3^{+}$ vacuoles (that reportedly are not generated in the context of chaperone-mediated autophagy), implying that it was specific for macroautophagy and failed to detect chaperone-mediated autophagy. We have mentioned this shortly in the corresponding part of the "Results and discussion" section.

2. *On page 10 the functional assays in supplemental Fig. 7 B and C should not be supplemental.*

   Our response: In strict compliance with the reviewer's desiderata, Supplemental Figures 7B and C have been advanced to Main Figures. In the revised version of the article, Supplemental Figures 7A-C figures have been included as Figures 5C-E, respectively. The remaining Figures have been rearranged accordingly. In particular, Figures 5C-G now constitute Figure 6 (A-E).

3. *On page 10, what is the functional consequence to starvation or rapamycin treatment to the IKK-beta knockout livers that don't induce efficient autophagy?*

   Our response: We reasoned that failure to activate autophagy in response to starvation should induce a reduction in circulating glucose and amino acids (because a general, whole-body autophagy defect causes death in response to starvation) (please see Komatsu M *et al.* J Cell Biol, 2005. 169:425-34; Kuma A *et al.* Nature, 2004. 432:1032-6.). However, we failed to detect a significant decrease in circulating glucose in the context of the liver-specific autophagy defect, in response to starvation. As a possibility, the liver-specific knockout of an essential autophagy inducer cannot cause a general metabolic defect because other organs (skeleton muscle, myocardium, kidney, etc…) that are induced into autophagy suffice to avoid starvation-induced morbidity. This possibility has been shortly discussed in the "Results and Discussion" section.

Additional Editorial Correspondence 05 November 2009

Many thanks for sending the revised version of your manuscript. I have now had the chance to look through it and your response to the reviewers' comments carefully, and I am pleased to tell you that we should be able to accept it for publication without sending it out for re-review. However, before we can do so, I noticed a problem with one of the figures: in Figure 2A, you appear to have included the wrong panel for the p62 staining under starvation conditions - instead, there is a duplicate of the LC3-GFP image.

I guess this was just an error in assembling the figure, and I suggest that the easiest way to deal with this would be if you could send us an updated version of the figure (as a tif like the original), which

we will upload in place of the previous version. Once we have this, we should then be able to accept the manuscript without further delay.

Additional Authors' Correspondence 06 November 2009

Please accept our apologies for the oversight. Enclosed please find a TIFF file corresponding to the correct version of Fig. 2.

We would be grateful if you could acknowledge the receipt of this e-mail, as well as the integrity and appropriateness of the enclosed file.

