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Che-1-induced inhibition of mTOR pathway enables stress-induced autophagy

Agata Desantis, Tiziana Bruno, Valeria Catena, Francesca De Nicola, Frauke Goeman, Simona Iezzi, Cristina Sorino, Maurilio Ponzoni, Gianluca Bossi, Vincenzo Federico, Francesca La Rosa, Maria Rosaria Ricciardi, Elena Lesma, Paolo D'Onorio De Meo, Tiziana Castrignanò, Maria Teresa Petrucci, Francesco Pisani, Marta Chesi, P. Leif Bersagel, Aristide Floridi, Giovanni Tonon, Claudio Passananti, Giovanni Blandino and Maurizio Fanciulli

Corresponding author: Maurizio Fanciulli, Regina Elena Cancer Center

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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.)

Editors: Alexander Kohlmaier, Andrea Leibfried

1st Editorial Decision 10 October 2014

Thank you for submitting your manuscript "Che-1 promotes multiple myeloma cell growth by inhibiting mTOR pathway and sustaining autophagy in response to stress." for consideration to the EMBO Journal. We have now received comments of two expert referees that you will find pasted below.

The two referees concur that your work is of general interest in principle, but some major conceptual as well as technical issues remain to be decisively addressed by experimentation before publication can be offered. Therefore, we invite you to revise the manuscript according to the referees' suggestions, which we consider very constructive.

Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. I will not repeat here in detail the referees' specific requests concerning mechanistic, and conceptual points and technical clarifications, but highlight only some key points for the revision.

One key issue in this respect for the revision is to directly and more compellingly determine autophagic flux by multiple accepted standard assays (Referee #1 point 7) in cells as well as tissues.

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Furthermore, other detailed functional experiments are suggested to substantiate the proposed regulation of autophagy (points 5,6).

Secondly, it will be essential to clarify how increased as well as decreased Che-1 levels activate Akt (point 3), as well as to more comprehensively assay mTORC2 kinase activity and activity of mTORC2 targets, in order to clarify how mTORC1 signaling is regulated (point 2).
Thirdly, the present study does not yet succeed in conclusively demonstrating the relevance of the proposed mechanism (Che-1-dependent regulation of mTOR for control of autophagic flux) in vivo, that is in the context of tumor cell survival. For the manuscript to be a strong candidate for publication we think that during revision, the effect of inhibition of mTOR signaling on cell death rates following Che-1 depletion should be functionally tested. Referee #1 offers a straight-forward approach (point 1).

Regarding referee #2's point 2, and also given referee #1's concerns that the IHC data are not easily interpretable as shown, we think that during revision the phenotype should be re-analyzed (in a more granular way?), quantified and better visualized to be able to better interpret the possibly incomplete penetrance. We also find referee #2's point 3 technically rather straight forward, which will certainly help to increase the potential impact of the paper.

Together, I am certain that also the other modifications requested by the referees would result in an improved study. I would, therefore, be pleased if you invested the necessary time and efforts to address the reviewers' concerns.

Should you have questions regarding the decision or should you have comments about extent or feasibility of a specific referee point, please do contact me.

I look forward to receiving your revision.

REFEREE COMMENTS

Referee #1:

In this study, the authors assess the effects of the stress response transcription factor Che-1 on mTOR signaling. Che-1 depletion is found to increase both Akt and mTORC1 signaling. Overexpression of Che-1 inhibits mTORC1 signaling, but not in cells lacking TSC2. Che-1 knockdown dampened the inhibitory effects of irradiation, hypoxia, and 2-deoxyglucose (2DG) on mTORC1 signaling. The transcript and protein levels of Redd1 and Deptor, two negative regulators of mTORC1, were decreased by Che-1 knockdown, and Che-1 associates with the promoters of these genes. Overexpression of either Redd1 or Deptor blocked the ability of Che-1 depletion to activate mTORC1 signaling. Evidence is provided that Che-1 might be required for hypoxia and 2DG to induce autophagy and that this is mediated through Redd1 and Deptor. Che-1 and, as described in previous studies, Deptor are found to be elevated in multiple myeloma (MM) samples, apparently correlating in their expression. Knockdown of Che-1 or Deptor was found to decrease Akt-S473 phosphorylation and induce apoptosis in two MM lines, and Che-1 knockdown blocked tumor growth in a xenograft model with one of these lines.

The cell biological data presented in this study on Che-1 and its effects on mTORC1 signaling through Redd1 and Deptor are interesting and compelling. However, the causal link between these findings and the second half of the story demonstrating the importance of Che-1 in multiple myeloma cell survival is only loosely made, despite the grossly overstated title. In addition, the authors' conclusion, as also stated in the title, that Che-1 is required for multiple myeloma cells to sustain autophagy is not rigorously supported by the data presented, which rely exclusively on a flawed readout of autophagy. There are also major discrepancies within the study regarding data on Che-1 and the regulation of Akt, which the authors conclude reflects effects on mTORC2 signaling.

Specific Comments:
1. The title is not appropriate and must be changed. This study certainly does not demonstrate that "Che-1 promotes multiple myeloma cell growth by inhibiting the mTOR pathway". The suggestion

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is that treatment with mTOR inhibitors would protect myeloma cells and tumors from death induced by Che-1 knockdown, which is never tested.

2. As Akt lies upstream of mTORC1 signaling, the data in Figure E1C showing that Akt is also activated by Che-1 depletion should be included in Figure 1, as it is an important finding when considering the potential mechanisms by which mTORC1 is influenced by Che-1. Importantly, it cannot be concluded from the experiment in E1C that Che-1 is affecting mTORC2 without measuring other mTORC2 targets and mTORC2 kinase activity. The levels of Akt phosphorylation on both T308, which is not assessed, and S473 in cells is predominantly a reflection of the activation status of PI3K, an increase in which would also explain the activation of mTORC1 signaling.

3. There is a major discrepancy in the results on Akt signaling, with Figure E1C showing that Che-1 knockdown activates Akt and Figure E5A showing that Che-1 overexpression also activates Akt in the same cell line (HCT116), with these contradictory findings not addressed.

4. Figure 2A: While the phosphorylation of Che-1 tracks with its protein levels, it should be determined whether the induction of Che-1 in response to hypoxia and 2DG occurs at the transcript level. If it really does required ATM and/or Chk1 in response to these specific stresses, then that should be demonstrated experimentally.

5. Figure 5/7E/8E: Che-1 depletion is decreasing the levels of both LC3-I and -II in these experiments, suggesting a more general effect on this protein rather than specific effects on the initiation of autophagy. The authors should attempt to explain this. The effects of Che-1 knockdown on LC3 (MAP1LC3B) transcript levels should be determined.

6. Do mTORC1 inhibitors (e.g., CCI-779 or Torin1) overcome the apparent block in autophagy observed upon Che-1 knockdown, assayed using the approach in Figure 5D/E.

7. In all of the studies on multiple myeloma samples and cells (Figures 6-8), the authors use LC3B-II levels as their only readout of autophagy. This is not a valid approach to assess autophagy, as increased levels of this marker could just as easily reflect a decrease in autophagic flux. Autophagy must be assessed by using the accepted standards in the autophagy field (see Klionsky et al., Autophagy 2012), preferably with multiple distinct assay. This is essential to the strong conclusions made in this study.

8. Figure 8D: As presented, this IHC data are not interpretable.

Minor:
1. Intro: Paragraphs 1 and 2 - Several inaccuracies are present. a) The statement that mTORC2 regulates the actin cytoskeleton and cell spreading, as if these were its most established functions, is incorrect. b) Tuberous sclerosis complex (TSC) is the name of a disease. The protein complex made up of TSC1 and TSC2 should be called the TSC1-TSC2 complex or the TSC complex. Also, it is NOT a GTPase, as stated, but a GTPase-activating protein (or GAP) for Rheb, which is the GTPase. c) Also, the citation provided for this finding (Inoki 2002) is not correct, as Rheb was not discovered until 2003. It is recommended that either all of the relevant citations around this be provided, or just a review article or two. d) The comments on S6 kinase enhancing the translation of specific mRNAs has no scientific basis, especially for ribosomal and mitochondrial biogenesis and oxygen consumption, as stated. e) In both paragraphs 1 and 2, the terms mTORC1 or mTORC2 should be used when discussing the regulation and targets of mTOR, as these are two distinct entities. It is recommended that this entire section be rewritten with an eye toward accuracy and a critical assessment of the scientific literature. It is also worth including more intro into the literature on Redd1 and Deptor, which become major components of this story. Much of this material can then be removed from the results section.

2. Figure 1C/D: Which cells are overexpressing myc-Che-1, while evident if one assesses the data, should be labeled in the panels.

3. Results, section 2, paragraph 1 - The extended content of this paragraph is redundant, and in many cases identical, to the introduction and should be restricted to describing experimental results. The text of the results section, in general, can be shortened significantly.

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4. The ChIP-seq data in Figure 3C should be presented in a way that is more meaningful and readily accessible to the reader.

Referee #2:

In the manuscript by Desantis et al., the authors continue their studies of the role of Che-1 in cell growth and survival. Here they focus on the role of Che-1 in stress responses and show that it regulates mTOR function through the induction of Redd1 and Deptor. Through up regulation of Redd1 and Deptor, Che-1 allows for autophagy to be sustained under stress conditions. Since Deptor has been linked to progression of myeloma in certain subtypes of this disease they investigated the role of Che-1 in myeloma. While they did not find an association with the Deptor-expressing Maf subtype, they did find that Che-1 was expressed in myeloma and based on gene expression studies demonstrated that it increases with progression. Functional in vitro and in vivo studies show a role for Che-1 in myeloma cell growth and survival.

Overall the studies are well performed and the data convincing. The findings are also novel as they identify a potential target that could make current therapies work better by preventing the protective stress-induced autophagy. Several concerns should be addressed.

Major Concerns

1. There is almost no statistical analysis of any of the data. This is particularly important for Figure 7D as it is not clear that siChe1 is inducing significant cell apoptosis, based on the error bars.

2. Only about half the symptomatic patient samples and about half the cell lines express Chi and Deptor. Even less of the relapsed samples. Why is this pathway only required in about half the samples assayed? These samples appear to have more autophagy not less which would be inconsistent with the authors' model.

3. The authors should investigate the effects of Che-1 silencing on therapeutic responses in myeloma, especially for proteasome inhibitors.

Minor Concerns

4. The title focuses on myeloma, however most of the experiments including all the mechanistic ones were not performed in myeloma. The title should more accurately reflect the contents of the manuscript.

5. The untreated controls in Figure 5C are not appropriate. Figure 5A shows that transfection, even with the siControl induces autophagy (1.8 fold change in LC3/Actin ratio). Therefore the ratios in 5C may over-estimate the effects of Chi-1 silencing.

1st Revision - authors' response 12 January 2015

We thank the reviewers for their critical reading and suggestions in reviewing our manuscript. We added relevant edits to the text and figures. These changes have much improved our manuscript.

Referee #1:

1. The title is not appropriate and must be changed. This study certainly does not demonstrate that "Che-1 promotes multiple myeloma cell growth by inhibiting the mTOR pathway". The suggestion is that treatment with mTOR inhibitors would protect myeloma cells and tumors from death induced by Che-1 knockdown, which is never tested.

We agree with this reviewer that the results presented do not completely demonstrate that *"Che-1 promotes multiple myeloma cell growth by inhibiting the mTOR pathway"*, even if we showed that Che-1 depletion affects multiple myeloma cell growth and mTOR activity by controlling Deptor expression (figs. 7B, 7C, E7E and E7F). Moreover, following the reviewer's suggestion, we treated Che-1 depleted myeloma cells with the mTORC1 inhibitor CCI-779, demonstrating that these treatment were able to rescue both in vitro and in vivo the death induced by Che-1 knockdown (figs. 8B, 8C, 8D, E7D, and E7L). However, in accordance with reviewer's request, we have changed the title of the manuscript.

2. As Akt lies upstream of mTORC1 signaling, the data in Figure E1C showing that Akt is also activated by Che-1 depletion should be included in Figure 1, as it is an important finding when considering the potential mechanisms by which mTORC1 is influenced by Che-1. Importantly, it cannot be concluded from the experiment in E1C that Che-1 is affecting mTORC2 without measuring other mTORC2 targets and mTORC2 kinase activity. The levels of Akt phosphorylation on both T308, which is not assessed, and S473 in cells is predominantly a reflection of the activation status of PI3K, an increase in which would also explain the activation of mTORC1 signaling.

As requested by this reviewer, we included fig. E1C in Figure 1 (fig.1B of the revised version). Moreover, to confirm that Che-1 depletion affects mTORC2 activity, we measured the phosphorylation levels of PKCa and SGK, two other mTORC2 targets (figs.1B and E6A).

3. There is a major discrepancy in the results on Akt signaling, with Figure E1C showing that Che-1 knockdown activates Akt and Figure E5A showing that Che-1 overexpression also activates Akt in the same cell line (HCT116), with these contradictory findings not addressed.

These opposite effects of Che-1 on mTORC2, similar to those described for Deptor (Peterson et al., Cell 2009; Yang et al., Genes and Cancer 2014) and Redd1 (Jin et al., Cancer Lett. 2013), can be explained considering that, because Che-1 overexpression inhibits mTORC1, it relieves the inhibitory feedback signal normally transmitted from mTORC1 to PI3K. Consistent with these observations, we found that AKT phosphorylation does not decrease when mTORC1 activity is suppressed by using the rapamycin analog CCI-779 (figs. 8D and E7D).

4. Figure 2A: While the phosphorylation of Che-1 tracks with its protein levels, it should be determined whether the induction of Che-1 in response to hypoxia and 2DG occurs at the transcript level. If it really does required ATM and/or Chk1 in response to these specific stresses, then that should be demonstrated experimentally.

In accordance with the reviewer's request, we performed qRT-PCR experiments to assess whether the Che-1 transcription is modulated in response to cellular stress. As shown in fig. E2A, we did not observe significant modifications of Che-1 mRNA following cellular stress. On the contrary, consistent with the finding that Che-1 phosphorylation by checkpoint kinases is required for its stabilization (Bruno et al., Cancer Cell 2006), the specific inhibition of ATM kinase activity produced a decrease of phosphorylation of Che-1 as well as its protein levels (fig. E2B).

5. Figure 5/7E/8E: Che-1 depletion is decreasing the levels of both LC3-I and -II in these experiments, suggesting a more general effect on this protein rather than specific effects on the initiation of autophagy. The authors should attempt to explain this. The effects of Che-1 knockdown on LC3 (MAP1LC3B) transcript levels should be determined.

Following the reviewer's suggestion, we performed qRT-PCR experiments to assess whether Che-1 affects MAP1LC3B mRNA expression. As shown in fig. E5A, we observed in concomitance of Che-1 depletion, a little decrease of MAP1LC3B mRNA, as also observed in the microarray analysis performed (not shown). However, a quantification analysis of LC3B-1 (LC3B-1/b-actin ratio) revealed that the LC3B-1 decreases observed in Che-1 depleted cells were always lower than LC3B-II (not shown) leading to assume multiple controls by Che-1 on LC3B.

6. Do mTORC1 inhibitors (e.g., CCI-779 or Torin1) overcome the apparent block in autophagy observed upon Che-1 knockdown, assayed using the approach in Figure 5D/E.

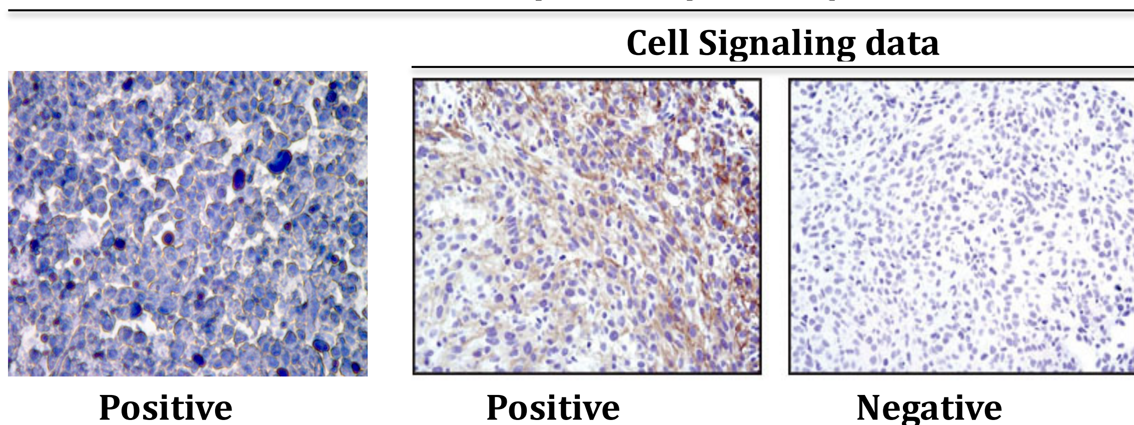
As requested by this reviewer, we evaluated whether CCI-779 treatment was able to overcome the autophagy block induced by Che-1 depletion. As shown in figs E5B, E5C and E5D, the mTORC1 inhibitor completely rescued the block in autophagy observed upon Che-1 knockdown.

7. In all of the studies on multiple myeloma samples and cells (Figures 6-8), the authors use LC3B-II levels as their only readout of autophagy. This is not a valid approach to assess autophagy, as increased levels of this marker could just as easily reflect a decrease in autophagic flux. Autophagy must be assessed by using the accepted standards in the autophagy field (see Klionsky et al., Autophagy 2012), preferably with multiple distinct assay. This is essential to the strong conclusions made in this study.

Following the reviewer's request we have utilized other approaches to evaluate autophagy levels in multiple myeloma cells. In particular, we performed immunofluorescence analyses of endogenous LC3B protein (figs E7A, E7K and E8E), and we evaluated p62/SQSTM1 expression in HCT116 and multiple myeloma cells in which Che-1 was depleted (figs. 5C, 8E and E7J).

8. Figure 8D: As presented, this IHC data are not interpretable.

Despite several efforts and the use of other batches of the anti-p-S6 and anti-p-Akt1 antibodies, we were not able to obtain ICH data of better quality (see figure attached below). For this reason in the revised manuscript we substituted fig.8D with a western blot analysis.



Minor:

1. Intro: Paragraphs 1 and 2 - Several inaccuracies are present. a) The statement that mTORC2 regulates the actin cytoskeleton and cell spreading, as if these were its most established functions, is incorrect. b) Tuberous sclerosis complex (TSC) is the name of a disease. The protein complex made up of TSC1 and TSC2 should be called the TSC1-TSC2 complex or the TSC complex. Also, it is NOT a GTPase, as stated, but a GTPase-activating protein (or GAP) for Rheb, which is the GTPase. c) Also, the citation provided for this finding (Inoki 2002) is not correct, as Rheb was not discovered until 2003. It is recommended that either all of the relevant citations around this be provided, or just a review article or two. d) The comments on S6 kinase enhancing the translation of specific mRNAs has no scientific basis, especially for ribosomal and mitochondrial biogenesis and oxygen consumption, as stated. e) In both paragraphs 1 and 2, the terms mTORC1 or mTORC2 should be used when discussing the regulation and targets of mTOR, as these are two distinct entities. It is recommended that this entire section be rewritten with an eye toward accuracy and a critical assessment of the scientific literature. It is also worth including more intro into the literature on Redd1 and Deptor, which become major components of this story. Much of this material can then be removed from the results section.

In complete agreement to the reviewer's considerations, we made all the text corrections requested.

2. Figure 1C/D: Which cells are overexpressing myc-Che-1, while evident if one assesses the data, should be labeled in the panels.

Following the reviewer's suggestion, we added Myc-Che-1 labels in these figures.

3. Results, section 2, paragraph 1 - The extended content of this paragraph is redundant, and in many cases identical, to the introduction and should be restricted to describing experimental results. The text of the results section, in general, can be shortened significantly.

As requested by this reviewer, we reduced the length of this paragraph.

4. The ChIP-seq data in Figure 3C should be presented in a way that is more meaningful and readily accessible to the reader.

We have presented fig.3C in a more accessible way.

Referee #2:

1. There is almost no statistical analysis of any of the data. This is particularly important for Figure 7D as it is not clear that siChe1 is inducing significant cell apoptosis, based on the error bars.

Following the reviewer's request, we have included statistical analysis in most graphics of the manuscript.

2. Only about half the symptomatic patient samples and about half the cell lines express Chi and Deptor. Even less of the relapsed samples. Why is this pathway only required in about half the samples assayed? These samples appear to have more autophagy not less which would be inconsistent with the authors' model.

The model proposed in this manuscript is that Che-1 induces autophagy by inhibiting mTORC1 activity in response to cellular stress. This result is achieved by activating Deptor and Redd1. Consistent with this model, autophagy levels in multiple myeloma samples strongly correlated with Che-1 and Deptor expression (figs. 6D, 6E, 6F and E6F). However, myeloma cells have demonstrated to be able to sustain autophagy and survival by several other mechanisms (Lamy et al., Cancer Cell 2013; Leung- Hagesteijn et al., 2013), and other important pathways sustain mTORC1 inhibition in multiple myeloma cells, promoting Akt1 activation and cell survival (Fernandez-Saiz et al., Nat. Cell. Biol. 2013). Therefore, it is not surprising that Che-1 and Deptor are found up-regulated only in about half of patients analysed, but it is very relevant that in cells expressing high levels of Che-1, its depletion induces cell death.

3. The authors should investigate the effects of Che-1 silencing on therapeutic responses in myeloma, especially for proteasome inhibitors.

As requested by this reviewer, we treated Che-1 depleted or not Kms27 cells with different doses of Bortezomib, a proteasome inhibitor largely utilized in the treatment of MM. From these experiments we found that Che-1 depletion in Kms27 cells strongly sensitized these cells to Bortezomib (fig. E7M), and similar results were obtained from Kms18 cells (not shown).

Minor Concerns:
4. The title focuses on myeloma, however most of the experiments including all the mechanistic ones were not performed in myeloma. The title should more accurately reflect the contents of the manuscript.

As requested by this reviewer, we changed the title of the manuscript.

5. The untreated controls in Figure 5C are not appropriate. Figure 5A shows that transfection, even with the siControl induces autophagy (1.8 fold change in LC3/Actin ratio). Therefore the ratios in 5C may over-estimate the effects of Chi-1 silencing.

We apologize for being not sufficiently clear on this figure. The "untreated" lanes indicated HCT116 cells transfected with siControl siRNA oligos but not treated with cellular stresses. Appropriate labels were added in the revised manuscript.

2nd Editorial Decision 29 January 2015

Thank you for submitting your revised manuscript for our consideration. Please note that Dr. Kohlmaier is not a member of our editorial team anymore, and I am now the handling editor for your manuscript.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #1's suggestion and to provide a final version of your manuscript.

REFEREE COMMENTS

Referee #1:

This is a vastly improved manuscript that is more balanced in its conclusions, which are now more thoroughly supported with data.

Final suggestion:
Given that the new panels in Figures E5B-D are the only ones supporting the primary conclusion, as stated in the title, that it is specifically Che-1's affect on mTORC1 that influences autophagy, these data should be moved to Figure 5.

Referee #2:

Previous concerns with the manuscript have been adequately addressed.

2nd Revision - authors' response 12 February 2015

Please find attached the final form of the manuscript "Che-1-induced inhibition of mTOR pathway enables stress-induced autophagy".

Following referee #1's suggestion, we have moved figure E5 to figure 5.

In addition, we have modified the sections that needed slight re-wording and checked the resolution of all figure files.

We believe that the old title could be a good summary "blurb" of our paper:

> "Che-1 promotes multiple myeloma cell growth by inhibiting mTOR pathway and sustaining autophagy in response to stress."

We therefore look forward to your response.

3rd Editorial Decision 16 February 2015

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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