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Acetylation of p53 stimulates miRNA processing and determines cell survival following genotoxic stress

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

1st Editorial Decision 24 May 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express great interest in the findings reported in your manuscript; however they also raise a number of concerns that you will need to address in full before they can support publication of the manuscript. I would like to emphasize that we agree with ref #2 that you need to strengthen the physiological significance of your findings by

- employing additional (tumor) cell lines
- addressing the effect of hMOF KD in a p53 expressing cell line
- establishing the functional requirement for hMOF and the p53 K120 acetylation in apoptosis

Understanding the chain of events leading to activation of hMOF following CPT treatment would certainly also strengthen the manuscript but is not an absolute requirement from our side. However, you should extensively discuss/elaborate on the potentially implicated pathways.

In addition, all three refs raise a number of additional points that should be addressed before your submit a revised version of your manuscript. We would also suggest that you follow the recommendation by ref#2 and restructure the manuscript with a clearer emphasis on the more conceptually novel findings.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised

version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

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, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

REFEREE REPORTS

Referee #1

This manuscript reports a very interesting set of findings concerning the function of p53 on microRNA biogenesis. The finding that lack of acetylation of K120 results in dysfunction of microRNA biogenesis without affecting the transcriptional activity of p53 is quite interesting. I believe that the manuscript is suitable for publication in EMBO J after minor revision.

Comments
1. Fig. 1B: They examined the effects of CPT and Nut3 only on Bcl-w, PUMA, and BAX. There are other Bcl-family pro-apoptotic and anti-apoptotic proteins. They should describe why they focused only on Bcl-w, PUMA, and BAX.
2. Processing targets of p53 include miR-16, 103, 143, 203, and 206, and miR-203 appears to be most important for repression of Bcl-w expression. The manuscript is difficult to understand for readers in several places, since they examined the effects of p53 on miR-203 in some experiments, but on other microRNAs in other experiments. For example, they should check the effects of si-hMOF on pre-miR-203, in addition to miR-103 and 143, in Fig. 5C.
3. They used only DXR in Fig. S3. Does CPT also show similar effects in p53(+) and (-) cells?

Minor points
1. Page 3: The first and second sentences are redundant.
2. Page 8: Fig. 2B bottom panel (line 2 and 3) should be Fig. 2B middle panel, and Fig. 2B middle panel (line 9) should be Fig. 2B bottom panel.
3. Page 10: Fig. 3A middle panel (line 8) should be Fig. 3A bottom panel, and Fig. 3A bottom panel (line 11) should be Fig. 3A middle panel.

Referee #2

In this manuscript, Chang et al. describe a novel mechanism that determines p53 response to stress-specific cellular outcomes, via miRNA processing. The authors compare the effect of different stimuli such as genotoxic agents and the small molecule Nut3, and find that p53 is involved in differential responses depending on the primary stimulus. They show how, in response to genotoxic inputs but not Nut3, K120 acetylation of p53 mediates the processing of a set of p53-targeted miRNAs by regulating the Drosha complex. The authors claim that in these particular situations, induction of miR203 prevents Bcl-w activation, thus leading to apoptotic cell death. In summary, they suggest that hMOF-dependent acetylation of K120 residue of p53 enhances binding to the Drosha complex and is critical for the processing of miRNAs. This work is in general properly performed and the authors include important controls throughout the manuscript to validate the data.

Most of the single molecular connections described in this work had been previously published, and the authors here integrate them to establish a novel mechanism of p53-mediated apoptosis; i.e. genotoxic stress leads to MOF induction, p53-acetylation at K120, increased miRNA levels, decreased Bcl-w and apoptosis. In general, this mechanism is of high interest and explains some previous observations on the differential response of p53 to different stimuli. Yet, the manuscript would benefit from a more focused presentation of the data that are novel.

Major points

1. Relevance of hMOF. hMOF is shown to be responsible for the K120 acetylation of p53 upon CPT treatment thus uncovering a critical role of K120 acetylation in regulating the miRNA biogenesis pathway and in determining a stress-specific cellular outcome. However, no upstream mechanisms are explored or even speculated. The authors could investigate further the signaling leading to hMOF activation upon CPT treatment. Is hMOF similarly induced by different genotoxic mechanisms leading to p53 induction (radiation, DNA damage, replicative stress?)

2. In addition, to what extent is hMOF sufficient to complement nutlin in achieving a full (CPT-like) response in terms of transcription targets, miR processing of several targets, Bcl-w levels and induction of apoptosis (and cell cycle arrest? Similar extended studies should be performed with K120Q: a comparison of transcriptional, miRNA targets, Bcl-w levels and comparison of cell cycle arrest versus apoptosis. In general, Bcl-w levels and apoptosis should be included as a major readout in most figures.

3. In Figure 5 the authors study the importance of Acetylation K120 of p53 by hMOF in the miRNA processing. They show different data to address this issue. However, most of the data shown in this figure is performed in p53 null cells. It would be interesting to analyze whether hMOF knockdown through siRNA inhibition in p53 (+) cells also leads to the abolishment of the CPT-induced miRNA processing. In addition, it would be valuable to analyze the ability to mediate apoptotic cell death upon knockdown of hMOF in response to genotoxic damage.

4. Only one cell line has been used in the whole study. The authors should demonstrate the mechanism described in additional cell lines and perhaps in some tumor-derived cell lines, for instance, any of those in which the K120R mutation has been observed. It would be highly relevant to test the oncogenic potential of this mutation in terms of miRNA processing and induction of apoptosis in these tumor-derived cells. Are those cells more resistant to chemotherapy?

Other considerations:

1. Do the authors suggest (Fig1B) that silencing of Bcl-w in Nut3-treated cells would lead to a caspase activation similar to that obtained upon CPT treatment?

2. In the discussion, the authors speculate about the role of p53 K120 acetylation not only in the repression of Bcl-w, but also in the induction of Bax and Puma. However, they just analyze the effects on Bcl-w repression and pay no attention to the levels of Bax/Puma (Figure 4). In Figure 3A, the levels of Bax and Puma induced by the different treatments are low. How do the authors explain these data? As indicated above, the induction of Bax and Puma with the K120R mutant in Figure 4 or the K120Q mutant in Figure 5 should be checked. Also, as stated above, the effect of hMOF overexpression/silencing should also tested.

3. The in vitro assay with biotin -labeled pri-miR203 is an interesting experiment that could be repeated in Figure 4 including the p53 K120R mutant or in Figure 5 including both the K120R and the K120Q mutants.

4. An expression construct of Bcl-w lacking the 3-UTR is used to mimick lack of miR-203 effect. However, a mutant in the specific miR-203 recognition element (as published in the original paper) should be desirable to discard the effect of other miRNAs or 3-UTR-binding proteins in that construct.

5. It is shown that acetylation of K120 facilitates stable interaction with p68 and Drosha. In figure 6

it can be seen that the acetyl K120 mimic K120Q was constitutively associated with p68 and Drosha in the absence of CPT treatment, while WT p53 associated with p68 only upon DNA damage stress. Is it the mutant K120R able to associate with p68 upon DNA damage? Data for this mutant is only shown without DNA damage stress (Figure 6B).

Minor comments:

1. The sentence "changes and can damage macromolecules, including DNA, mRNA, proteins, and lipids, which need to be replenished" is duplicated in the first lines of introduction.

2. "As a control of specificity, miRNAs regulated by the signal transducers of the TGFβ signaling pathway, Smad proteins (miR-21, miR-199a, and miR-421), have been examined after DXR treatment and were not affected by the activation of p53 by DXR (Supple Fig. S3B), indicating the specificity of miRNAs that are regulated by p53 upon DXR or CPT stimulation." These data are not included in that Supp Figure.

3. "Therefore, we hypothesized that CPT but not Nut3 mediates the acetylation of K120 of p53, which leads to the induction of miR-203 and subsequent cell death" (first paragraph in page 10). It is not clear this first reference to K120 in this paragraph which is mostly focused to miRNAs..

4. "suggesting that miR-203 functions as a tumor-suppressor12,14,28-33" (page 17). Citations should read: "12-14,28-33"

5. Fig. 5C. Does miR-103 refers to "miR-203"?

Referee #3

Chang et al.

Role of lysine acetylation of the p53 DNA-binding domain in the regulation of microRNA processing

In this manuscript, the authors have analyzed the role of p53 in the regulation of cell death upon DNA damage induction. They show that the DNA damage inducing molecule camptothecin (CPT) induces apoptosis by down regulating the anti-apoptotic protein Bcl. Bcl is a direct target of miR-203 and therefore miR-203 induction leads to Bcl down regulation and cell death. In further experiments, the authors show that the miRNA-processing stimulation activity is specifically activated upon CPT treatment while other activities of p53 remain unaffected. Stimulation of miRNA processing by p53 is achieved by K120 acetylation mediated by the acetyltransferase hMOF. Finally, the authors show that acetylation at K120 facilitates the interaction between p53 and p68, which interacts with Drosha.

This is a well-performed study. It is well written and the results are presented clearly. I have only a few points that need clarification.

1. What is amplified in Figure 3D? I guess the pre-miRNAs? Or pri-miRNAs? It is not clearly stated in the Figure legend.

2. The first sentence of the introduction is duplicated and should be removed.

3. Figure 6A: many western blots differ in quality. For example, the upper p68 western blot is rather over exposed and the band is not visible. The lower one in the same Figure looks much better. This holds true for several p53 western bots as well.

4. Figure 3D: the p53 blot shows a double band? How can this be explained? In other p53 western blots, a second band is not visible.

Authors' response to the referees

Referee #1

We would like to thank Referee#1 for stating that our manuscript reports a very interesting set of findings concerning the function of p53 on microRNA biogenesis and that it is suitable for publication after minor revision.

1. Fig. 1B: They examined the effects of CPT and Nut3 only on Bcl-w, PUMA, and BAX. There are other Bcl-family pro-apoptotic and anti-apoptotic proteins. They should describe why they focused only on Bcl-w, PUMA, and BAX.

We chose to focus on the pro-apoptotic molecules Puma and Bax because they are wellcharacterized transcriptional targets of p53, and are also essential for p53-mediated apoptosis. We have examined three members of the Bcl-2 family of pro-survival genes: Bcl-w, Bcl-2 and Bcl-x. Only Bcl-w was specifically regulated by CPT but not by Nutlin. According to the referee's comment, the text was revised.

2. They should check the effects of si-hMOF on pre-miR-203, in addition to miR-103 and 143, in Fig. 5C.

According to the referee's advice, we included data on other p53-dependent miRNAs, such as miR-16 and miR-203, in Fig. 5C.

3. They used only DXR in Fig. S3. Does CPT also show similar effects in p53(+) and (-) cells?

The effects of CPT and DXR in p53(+) cells in terms of (i) induction of p53 and (ii) processing of miRNAs are very similar; the data are shown in Fig. 3A. In Suppl. Fig. S4, we include a side-by-side comparison of the effect of different genotoxic agents, such as CPT, DXR, actinomycin D (ActD), and etoposide (ETP) in p53(-) cells.

4. Page 3: The first and second sentences are redundant.
The text was revised.

5. Page 8: Fig. 2B bottom panel (line 2 and 3) should be Fig. 2B middle panel, and Fig. 2B middle panel (line 9) should be Fig. 2B bottom panel.
The figure format was revised.

6. Page 10: Fig. 3A middle panel (line 8) should be Fig. 3A bottom panel, and Fig. 3A bottom panel (line 11) should be Fig. 3A middle panel.
The figure format was revised.

Referee #2

We would like to thank Referee#2 for commenting on the experiments in our manuscript being in general properly performed, on the inclusion of important controls throughout the manuscript to validate the data, and on the mechanism we proposed being of high interest and useful to explain previous observations on the differential response of p53 to different stimuli.

1. Is hMOF similarly induced by different genotoxic mechanisms leading to p53 induction (radiation, DNA damage, replicative stress?)

A previous study by Sykes *et al*. (Molecular Cell, 2006) demonstrates no significant change in hMOF expression upon genotoxic stress. Our result included in Suppl. Fig. S8 agrees with the observation by Sykes *et al*. and indicates that the hMOF protein level is

unchanged upon CPT treatment.

2. Studies, such as a comparison of transcriptional, miRNA targets, Bcl-w levels and comparison of cell cycle arrest versus apoptosis, should be performed with K120Q.

Based on the referee's advice, we expanded the characterization of the p53-K120Q mutant. These results are included in Fig. 5E (Puma and Bcl-w), Fig. 5F (apoptosis assay), and Fig. 6C (in vitro pri-miRNA processing assay). The highlight of these data is Fig. 6C, showing that the pri-miR-203 processing activity of K120Q-p53 is constitutively higher than wild type p53.

3. In Figure 5, it would be interesting to analyze whether hMOF knockdown through siRNA inhibition in p53 (+) cells also leads to the abolishment of the CPTinduced miRNA processing. In addition, it would be valuable to analyze the ability to mediate apoptotic cell death upon knockdown of hMOF in response to genotoxic damage.

We performed the experiments suggested by the reviewer, and the results are presented in Suppl. Fig. S7 and Fig. 5B-D. Downregulation of hMOF inhibits: CPT-dependent induction of pri-miR-203 processing (Fig. 5C and Suppl. Fig. S7), downregulation of *Bclw* (Fig. 5C), and induction of apoptosis (Fig. 5D). On the contrary, overexpression of hMOF mediates a weak activation of caspase-3/7 (Fig. 5B). These results support our conclusion that hMOF-dependent K120 acetylation of p53 is critical for the induction of miRNA processing and promoting cell death upon CPT treatment.

4. Only one cell line has been used in the whole study. The authors should demonstrate the mechanism described in additional cell lines and perhaps in some tumor-derived cell lines, for instance, any of those in which the K120R mutation has been observed. It would be highly relevant to test the oncogenic potential of this mutation in terms of miRNA processing and induction of apoptosis in these tumor-derived cells. Are those cells more resistant to chemotherapy?

We agree with the referee that understanding the mechanism of chemoresistance using tumor cells carrying the p53-K120R mutation would be clinically relevant and highly interesting. However, we have not found a cell line that bears exclusively the p53-K120R mutation. We have obtained cell lines, such as T24 and KYSE70, which were originally reported to harbor the p53-K120R mutation, but were subsequently found to carry much larger deletions in the p53 gene. While tumor cell analysis is currently unfeasible, an animal model for this mutation exists. A mouse bearing a homozygous p53-K120R "knock-in" mutation (K117R in mouse) has been generated; cells derived from these mice are unable to mediate apoptosis while they are capable of undergoing senescence and cell-cycle arrest upon p53 activation by gamma-irradiation (Li et al. Cell 2012). These results are consistent with our study.
To address the reviewer's concern that only HCT116 cells are used in our study, the H1299 human lung carcinoma cell line, which bears a homozygous deletion in the p53 gene similar to the HCT116 p53(-) cells, was subjected to CPT treatment. These results are presented in Suppl. Fig. S6.

5. Do the authors suggest (Fig1B) that silencing of Bcl-w in Nut3-treated cells would lead to a caspase activation similar to that obtained upon CPT treatment?

We performed the experiment suggested by the reviewer and present the result in Suppl. Fig. S1. The result indicates that Nut3 induces apoptosis similarly to CPT when *Bcl-w* is downregulated by siRNA.

6. The induction of Bax and Puma with the K120R mutant in Figure 4 or the K120Q mutant in Figure 5 should be checked.

According to the reviewer's advice, we performed these experiments and included the data on Bax and Puma in Fig. 4A and Fig. 5E. These results support the previously published result that K120R exhibits a slightly reduced induction of Puma and Bax, while the induction of p21 by CPT is not significantly affect. K120Q is unable to induce Puma, indicating that Ac-K120 is not sufficient for the transcriptional activation of Puma.

7. The in vitro assay with biotin-labeled pri-miR203 is an interesting experiment that could be repeated with the K120 mutants.
We performed the in vitro pri-miR-203 processing assay using cell lysates from K120Q or K120R mutant of p53. The result suggests that K120Q exhibits more efficient pri-miR-203 processing at the basal state compared to wild type p53, which is consistent with our conclusion that acetyl-K120 is sufficient to promote miRNA processing by the Drosha complex.

8. An expression construct of Bcl-w lacking the 3'-UTR is used to mimic lack of miR-203 effect. However, a mutant in the specific miR-203 recognition element (as published in the original paper) should be desirable to discard the effect of other miRNAs or 3-UTR-binding proteins in that construct.
According to the reviewer's advice, we performed the experiment with the luciferase construct containing full length 3'-UTR of Bcl-w mRNA with mutations in the miR-203 MRE sequence and included it in the revised Fig. 2E. The result indicates that both the 3'UTR-deleted construct and the construct with mutations in the miR-203 MRE are resistant to CPT treatment and unable to mediate cell death, confirming our conclusion that miR-203-mediated downregulation of *Bcl-w* plays a critical role in the CPT-mediated induction of cell death.

9. It is shown that acetylation of K120 facilitates stable interaction with p68 and Drosha. In figure 6 it can be seen that the acetyl K120 mimic K120Q was constitutively associated with p68 and Drosha in the absence of CPT treatment, while WT p53 associated with p68 only upon DNA damage stress. Is it the mutant K120R able to associate with p68 upon DNA damage? Data for this mutant is only shown without DNA damage stress (Figure 6B).
According to the reviewer's advice, we performed the experiment and presented it in Suppl. Fig. S8. The result suggests that K120R is unable to interact with p68 upon CPT stimulation, consistently with the result that K120R is unable to mediate miRNA processing upon CPT treatment.

10. The sentence "changes and can damage macromolecules, including DNA, mRNA, proteins, and lipids, which need to be replenished" is duplicated in the first lines of introduction.
The text has been corrected.

11. "As a control of specificity, miRNAs regulated by the signal transducers of the TGFβ signaling pathway, Smad proteins (miR-21, miR-199a, and miR-421), have been examined after DXR treatment and were not affected by the activation of p53 by DXR (Supple Fig. S3B), indicating the specificity of miRNAs that are regulated by p53 upon DXR or CPT stimulation." These data are not included in that Supp Figure.
The supplementary figure was accidentally omitted and has been restored in Suppl. Fig. S5.

12. "Therefore, we hypothesized that CPT but not Nut3 mediates the acetylation of K120 of p53, which leads to the induction of miR-203 and subsequent cell death" (first paragraph in page 10). It is not clear this first reference to K120 in this paragraph which is mostly focused to miRNAs.
According to the referee's suggestion, the text has been revised.

13. "suggesting that miR-203 functions as a tumor-suppressor12,14,28-33" (page 17). Citations should read: "12-14,28-33"

According to the referee's suggestion, all 8 references are cited in the revised text.

14. Fig. 5C. Does miR-103 refers to "miR-203"?
In the revised Fig. 5C, top panel, we included results for miR-16 and miR-203 in addition to miR-103 and miR-143.

Referee #3

We would like to thank Referee#3 for commenting that this is a well-performed study, well written, and the results are presented clearly.

1. What is amplified in Figure 3D? I guess the pre-miRNAs? Or pri-miRNAs? It is not clearly stated in the Figure legend.
They are pri-miRNAs. According to the referee's comment, Fig. 3D has been revised to clearly indicate "pri-miRs".

2. The first sentence of the introduction is duplicated and should be removed.
The text was revised.

3. Figure 6A: many western blots differ in quality.
We reloaded the same samples on an SDS gel and repeated the p68 western blot. The result is shown in the revised Fig. 6A.

4. Figure 3D: the p53 blot shows a double band?
Previously, a different antibody against p53 was used for the western blot. We reloaded the same samples on the SDS-PAGE and repeated the western blot, which is now shown in the revised Fig. 3D.

2nd Editorial Decision 13 September 2013

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below.

As you will see both referees find that the criticisms originally raised have been sufficiently addressed and consequently recommend your manuscript for publication; however, before we can proceed towards official acceptance of your work I need you to look into the following editorial points:

-> Provide figures with higher resolution, especially for depictions of gels and blots, in order to meet standards required for production.

-> Indicate number of replicas for statistical analysis as well as the nature of the error bars in figure legends (also for supplemental figures).

-> Reformat literature references according to the journal guidelines.

-> Include author Roberto Nessa in author contribution list and clarify whether author JL was the only person involved in performing experiments.

-> If possible, change scale for figures S4 and S5 to allow clearer visualization of fold induction

-> In addition, we now require authors to provide us with manuscript source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format uploaded as "Source data files". All gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary

"Source Data".

-> Lastly, I would encourage you to change the title for the manuscript in order to more clearly emphasize the functional outcome of p53 acetylation. A suggesting from our side could be: Acetylation of p53 stimulates miRNA processing and determines cell survival following genotoxic stress

Given the referees' positive recommendations, I would like to invite you to submit a final version of the manuscript, addressing all editorial points listed above. Please do not hesitate to contact me with any questions regarding this process.

Thank you for giving us the opportunity to consider your manuscript for The EMBO Journal. I look forward to your revision.

REFEREE REPORTS

Referee #2

The manuscript has significantly improved technically and all required controls are now included. This manuscript nicely integrates previous and novel observations on p53 response to specific stimuli and microRNA biogenesis. The regulation of hMOF remains to be established but this paper will hopefully set the basis for further studies.

Referee #3

The authors have addressed all points I had raised on the previous version of the manuscript. Therefore, I am satisfied with the revised version.

