EMBO reports

Manuscript EMBO-2015-41181

# A novel cytoprotective function for the DNA repair protein Ku in regulating p53 mRNA translation

Assala Lamaa, Morgane Le Bras, Nicolas Skuli, Sebastien Britton, Philippe Frit, Patrick Calsou, Hervé Prats, Anne Cammas, Stefania Millevoi

*Corresponding author: Stefania Millevoi, Inserm*

**Review timeline:**

| | |
|---|---|
| THE EMBO JOURNAL | |
| Submission date: | 23 July 2015 |
| Editorial Decision: | 10 August 2015 |
| Transferred to EMBO reports: | 13 August 2015 |
| EMBO REPORTS | |
| Editorial Decision: | 07 September 2015 |
| Revision received: | 18 December 2015 |
| Editorial Decision: | 08 January 2016 |
| Revision received: | 25 January 2016 |
| Accepted: | 27 January 2016 |

Editor: Esther Schnapp

## 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.)

THE EMBO JOURNAL 10 August 2015

Thank you for submitting your manuscript to The EMBO journal and my apologies for the delay in communicating our decision to you. I have now read your study carefully and discussed the work with other members of the editorial team as well as consulted with an external expert advisor. I am afraid the outcome of these discussions is that we have decided not to pursue publication of this manuscript. However, I have taken the liberty to also discuss your study with my colleague Esther Schnapp at EMBO Reports and she would be interested in offering peer review if you were to transfer your manuscript there.

From our side, we do appreciate that you show Ku depletion to trigger an increase in p53 protein expression and find that this effect derives from a direct interaction between Ku and a stem-loop segment in the 5' UTR of the p53 mRNA. You are furthermore able to show that either the induction of DNA damage or the acetylation of Ku causes the Ku70/Ku80 heterodimer to be released from p53 mRNA, thus relieving translational repression and increasing the potential for inducing apoptosis. However, we also had to notice that Ku has previously been shown to bind RNA stem loops in the context of telomerase and that post-transcriptional control of p53 expression can be exerted by several other known factors. In addition, we find that the broader functional context for this regulation remains rather open at the current stage.

 

Based on these concerns we consulted with an external expert advisor who gave us the following recommendation:

'My view is that this manuscript is better destined for EMBO Reports rather than EMBO Journal. One issue I have, is that because siRNAs have off-target effects on p53, the authors should really carry out complementation studies, especially given that they have access to cells expressing tagged wt and mutant Ku. Perhaps the authors ought to do these additional studies before the manuscript is considered for in-depth review.'

In conclusion, and in light of the input from our external expert advisor, we find that your current manuscript would be a much stronger candidate for publication in EMBO Reports than in EMBO Journal. While I am thus sorry to say that we have decided not to send the manuscript out for peer-review for The EMBO Journal, I would strongly recommend you to transfer the manuscript to EMBO Reports following the link provided below. I do want to add that my colleague there, Esther Schnapp, would ask you to include the complementation experiments suggested by our advisor before you submit the study there, in order to preempt similar issues from being raised by the referees.

Thank you for giving us the opportunity to consider this manuscript. I regret that we have to disappoint you on this occasion, but I hope that you will use this opportunity to transfer your work to EMBO Reports.

1st Editorial Decision 07 September 2015

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the full set of referee reports on your manuscript that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also all point out that quantifications and statistics are missing and required to support and strengthen the main conclusions. Importantly, the biological relevance of Ku-regulated p53 expression remains unclear. It will therefore be important to demonstrate that DNA damaging agents do not up-regulate p53 protein levels in the presence of the Ku acetylation deficient mutant K282A, as referee 3 suggests.

The editorial advisor consulted by my colleague Anne further notes that siRNAs have off-target effects on p53, and that rescue experiments must be performed with wild-type and mutant Ku to show that the effect is mediated by Ku. All missing controls also need to be added.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 expanded view figures. The results and discussion section must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file. For a normal article there are no length limitations, but it should have more than 5 main figures, the results and discussion section must be separate and the entire materials and methods included in the main manuscript file.

 

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This is an interesting study that associates acetylation-sensitive KU binding to a structured region of the 5' UTR of p53 with translational regulation. Overall, the data are clean and generally support the conclusions drawn. However, throughout many of the key figures there is a need for quantitative and statistical analyses of the data to document the reproducibility of the reported observations.

1. Fig. 1: is the upregulation of p53 statistically significant? Providing this analysis would give the reader confidence in the reproducibility of the data.
2. Fig. 1G: Is the ~1.3X increase in p53 mRNA levels statistically significant? If so, then it needs to be discussed more and perhaps incorporated into the final model developed for Ku regulation of p53 (particularly since many of the alterations in expression that are noted are likely in the ~2-3X range).
3. Fig. 2C: Can the 70/80 Kd cross linked proteins be immunoprecipitated with Ku antibodies? This would provide a definitive identification of the band.
4. Fig. 3C: since the effect appears to be subtle, please quantify these data to make them more convincing.
5. Fig. 4A and E: providing quantification/statistics would make these data fully convincing.

Referee #2:

This manuscript presents experimental evidence supporting the contention that the human Ku heterodimer (hKu) regulates the expression level of the p53 protein by attenuating translation of the p53 mRNA, and that this attenuation is suppressed by acetylation of hKu. Based largely on the results of RNA affinity chromatography utilizing biotinylated, in vitro transcribed RNA, the authors propose that this regulation is accomplished by hKu binding a bulged stem-loop in the 5' untranslated region (UTR) of p53 mRNA. It is further proposed that when hKu is acetylated, it no longer binds to this target, thereby eliminating the suppression of translation and allowing p53 to be expressed at higher levels.

This model is definitely intriguing and potentially important, and the data presented, much in the form of (usually qualitative) comparisons of western blots, appear consistent with it at the phenomenological level.

Fig. 1 shows that knockdown of Ku increases p53 expression levels. Because the disruption of DNA damage signaling might very well indirectly affect p53 levels, the authors do a number of experiments that lead to the hypothesis that the effect is direct. But they very appropriately understand that they need to show a direct connection between Ku and p53 to put their observations into a mechanistic framework.

 

Major points:

1.The specific model proposed for regulation, to wit hKu binding a bulged stem loop of RNA, is not yet convincing. The western blots of the eluates from RNA affinity chromatography are provocative and suggest the possibility of a specific interaction, but they are qualitative in nature.
When studies are done in cell extracts (Fig. 2A), bridging factors could participate in binding. A dramatically more convincing case could be made with quantitative binding data, such as an electrophoretic mobility shift assay (EMSA) with hKu and (non-biotinylated) in vitro transcribed RNA. They could test the nice set of RNAs shown in Fig. 2E (or some subset of these).

2.The hKu-RNA interaction model becomes more tenuous when considered in the context of the text on manuscript page 10, "Since Ku did not regulate translation of reporters in which the 5'UTR is attached to a luciferase ORF (data not shown), we concluded that the formation of this large hairpin domain including sequences within the p53 ORF is required for proper binding of Ku". If this is true, then if one further pursued the deletion experiments shown in figure 2D (i-ii) from the 3' end, there should be a point prior to the start codon position where deletion would abrogate binding. Or perhaps even easier, since they've shown that the elements in F4 are sufficient for Ku binding, why not attach F4 to a luciferase ORF?

3.The text is often difficult and frustrating to understand. A good example concerns Fig. 3C. (a) In part ii, is the "5'p53" perhaps a typo, and the authors really mean that the western uses an anti-p53 antibody? (5'p53 has a very specific meaning as defined In Fig. 2A.) (b) The control p53 blot of the cells without mRNA transfected is missing. (c) Most importantly, it's unclear whether there's really more p53 signal in the WT lanes upon knockdown of Ku70. How much difference is there, and could three biological replicates be shown?

Referee #3:

In the manuscript "A novel cytoprotective function for the DNA repair protein Ku in regulating p53 mRNA translation", the authors describe a novel role for the DNA repair protein-complex Ku in translational regulation. First, they showed that depletion of Ku via siRNA treatment in HCT116 cells resulted in an increased p53 expression without affecting overall DNA damage response pathways. Based on Takagi et al. (which demonstrated Ku binding to the 5´UTR of p53) the authors investigated the interaction of Ku with a stem-loop structure within the p53 5´UTR. Under normal conditions Ku is binding through a bulge motif in the 5´UTR stem loop structure to the p53 transcript and therefore repress p53 translation and further p53 downstream effects. DNA damage causes an acetylation of Ku, which results in the abrogation of the Ku - p53 5´UTR interaction.

These results not only suggest a novel aspect of Ku as RBP in post-transcriptional regulation but also its RNA-binding alteration via acetylation. Thus, this manuscript should be of interest for the DNA damage and the translational regulation field.
The results are mostly solid and convincing. However, there are some points that should be addressed in order to strengthen the manuscript before publishing:

- The effect of Ku overexpression. Since the authors precisely illustrate the effect of Ku depletion on p53 translation it would be favorable to see the reciprocal effect on p53 translation (with and without stress) by overexpressing the Ku-complex.

- Ku acetylation deficient mutant (Ku70 K282A): The authors showed a reduced binding of the constitutive acetylation mimicking Ku70 mutant in comparison to wildtype Ku. Since acetylation of K282 seems to be important, a mutant deficient for acetylation should invert the effect (and bind to 5´p53) and underlining the role of K282 acetylation in Ku binding to RNA. Furthermore, it would be interesting to see that DNA damage does not have the same effect on this mutant (K282A) than on wildtype Ku in terms of translational regulation of p53.

- Experimental controls:
A) UV cross-linking assay (Figure 2C): to support the statement "Ku binding to the 5´UTR is direct and specific" (page 7 line18) a negative control and a non-cross-linked control should be included (at least in the supplement). B) p53 translation /polysomal gradient: a negative control not shifting

 

from the sub-polysomal to polysomal fraction (also here in the supplement figures) after Ku depletion would further strengthen the data (although the comparison in Fig3b is done to the corresponding Input). C) Assay "p53mRNA as translational target of Ku" (Figure 3C): This assay nicely shows the effect of the B2loop in the 5´UTR on Ku mediated p53 translation. Nevertheless, loading controls and an indicated quantification of the western blot bands would be beneficial (same for Fig. 4A and 4C).

- Minor comments:
- Fig 4F: It would be great if the authors could include the difference of the two DMSO conditions. Since once the outcome is Ku acetylation and the other time not.
- Consistency in writing: DNA-damage vs DNA damage, crosslinking vs cross-linking or DNA-binding domain vs DNA binding domain.

1st Revision - authors' response 18 December 2015

Here is a point-by point response to the referee's comments:

***Fig. 1: is the upregulation of p53 statistically significant? Providing this analysis would give the reader confidence in the reproducibility of the data.***

As requested by the referee, we added the quantification of p53 upregulation after Ku silencing (Fig. 1A), showing that it is statistically significant.

*2.* ***Fig. 1G: Is the ~1.3X increase in p53 mRNA levels statistically significant? If so, then it needs to be discussed more and perhaps incorporated into the final model developed for Ku regulation of p53 (particularly since many of the alterations in expression that are noted are likely in the ~2-3X range).***

Indeed, Fig. 1G now shows that Ku depletion induces a slight but statistically significant increase in p53 mRNA levels, suggesting that p53 regulation by Ku might occur at multiple levels. This effect could explain the observation in Fig. 4A that p53 accumulation after Ku depletion during DDR was not completely lost after treatment with etoposide or bleomycin. However, we feel that this effect deserves to be thoroughly investigated before being included in our model.

***3. Fig. 2C: Can the 70/80 Kd cross linked proteins be immunoprecipitated with Ku antibodies? This would provide a definitive identification of the band.***

By using UV cross-linking assays followed by immunoprecipitation with an antibody recognizing the conformational epitope of the Ku heterodimer (Supplemental Fig. 3), we showed that the cross-linked complexes consisted indeed of Ku70/Ku80 bound to the p53 mRNA, supporting the notion that Ku-p53 mRNA interactions are the result of a direct contact between the Ku70/80 and the p53 RNA.

***4. Fig. 3C: since the effect appears to be subtle, please quantify these data to make them more convincing***.

Quantification of data in Fig. 3C (Fig. 3B (iii) in the revised version) now indicates that Ku depletion significantly increases p53 accumulation of the WT compared to the mutated DB2 reporter.

***5. Fig. 4A and E: providing quantification/statistics would make these data fully convincing.***

We now provide quantification and statistics of Fig. 4A, showing that p53 accumulation after Ku depletion is significantly reduced after treatment with etoposide and bleomycin.
Quantification/statistics of data in Fig. 4E (Fig. 4D in the revised version) show that the association of Ku with the p53 5'UTR is significantly reduced after treatment with bleomycin, TSA and ETO (Fig. 4D (ii), revised version).

 

Referee #2:

Major points:

***1. When studies are done in cell extracts (Fig. 2A), bridging factors could participate in binding…***

We agree this is an important point. As suggested by the referee, we performed EMSA with purified recombinant human Ku70/80 [1] to demonstrate that Ku binds to the p53 mRNA stem-loop structure in the absence of a bridging factor. Since our results (Fig. 2E, 3B, 3C in the revised version) demonstrated that the bulge sequence preceding the apical stem (Bulge 2 or B2) is important for Ku binding to the p53 mRNA and for its role in regulating p53 translation and function, we performed EMSA analysis using p53 RNA constructs containing the stem-loop structure WT (fragment F3, depicted in Fig. 2D) or mutated in the loop proximal bulge (DB2) (depicted in Fig. 2E). As a negative control, we used the RNA construct carrying the deletion of the loop distal bulge (DB1) that lost its ability to bind Ku (Fig. 2E). As shown in Supplemental Fig. 4A, the WT p53 RNA (F3) was shifted to a single lower mobility complex by the addition of purified recombinant Ku70/80. Increasing the amount of Ku70/80 resulted in the formation of at least two predominant slower migrating forms of ribonucleoprotein complexes with concomitant lost of free labeled p53 RNA. In agreement with affinity chromatography experiments (Fig. 2E), quantification of the fraction bound revealed that Ku binds the WT sequence with greater affinity than the DB2 mutant but with a similar affinity compared to the DB1 mutant. The specificity of binding was demonstrated further by **1**) competition gel shift analysis with unlabeled RNAs showing that unlabeled p53 RNA WT (F3) competes much better than the p53 RNA containing the DB2 mutation (Supplemental Fig. 4B) and **2)** substitution of Ku by purified recombinant GFP (negative control protein) (Supplemental Fig. 4B). Overall, these results demonstrate that Ku binds the p53 mRNA in the absence of bridging factors and provide a more quantitative evidence of the differential binding of Ku to the WT or mutated stem-loop region. We believe that it would be premature at this time to provide association/dissociation constants with enough precision due to: **1**) the formation of multiple shifted complexes at high Ku concentrations whose molecular nature cannot been defined at this stage **2**) EMSA has been performed with ≈200 nts RNA constructs (as in Fig. 2E) that might have a high propensity to improperly fold and thereby artificially increase the binding constants.

***2. Or perhaps even easier, since they've shown that the elements in F4 are sufficient for Ku binding, why not attach F4 to a luciferase ORF?***

We performed the experiments suggested by the referee but we could not observe a difference in the expression between the F4-luc and the F5-luc constructs (using Luciferase or 2CP Luciferase ORF) after siRNA-mediated depletion of Ku or Ku overexpression (data not shown). One possible explanation is that the p53 stem-loop is not formed in the luciferase reporter and therefore, that folding of the stem-loop requires the presence of the sequence contained in the p53 ORF. This possibility is confirmed by MFOLD prediction (data not shown) but deserves to be experimentally tested. Another important point is that the stem-loop contains the p53 AUG and therefore, translation of the luciferase reporter can be initiated at this AUG and/or at the luciferase AUG. In the case of the p53 reporter or the p53 mRNA, translation is mainly initiated at the p53 AUG but this might not be the case with the luciferase reporter. To definitively answer the referee comment, it would be worth to define whether initiation at the AUG embedded in the stem-loop structure plays a role in Ku mediated regulation of p53 translation.

3. ***Fig. 3C (a) In part ii, is the "5'p53" perhaps a typo, and the authors really mean that the western uses an anti-p53 antibody? (5'p53 has a very specific meaning as defined In Fig. 2A.) (c) Most importantly, it's unclear whether there's really more p53 signal in the WT lanes upon knockdown of Ku70. How much difference is there, and could three biological replicates be shown?***

We have now modified Fig. 3C (Fig. 3B in the revised version)

- **a-** To make clear that the p53 antibody was used to check p53 protein expression from the p53 reporter depicted in Fig 3B (i) (revised version) containing the 5'p53 region WT or mutated (DB2)

 

**b-** To include a loading control ((Fig 3B (ii), revised version) (asked by referee #3).
**c-** To quantify p53 accumulation upon Ku depletion and provide statistics indicating that p53 increases after Ku depletion in a significant manner only when expressed from the WT p53 reporter not from the DB2 reporter (Fig 3B (iii), revised version).

***(b) The control p53 blot of the cells without mRNA transfected is missing.***

We added the control requested by the referee in Supplemental Fig. 5C, showing that p53 expression in H1299 (p53-null) cells can be detected only when transfecting p53 reporters.

Referee #3:

***- The effect of Ku overexpression. Since the authors precisely illustrate the effect of Ku depletion on p53 translation it would be favorable to see the reciprocal effect on p53 translation (with and without stress) by overexpressing the Ku-complex.***

[Text and Figures were removed upon authors' request. Data was redacted as it may form part of a future publication.]

Based on these data, we feel that we cannot draw a definitive conclusion on the ability of ectopic Ku to regulate p53 translation, and therefore we did not include these results in the manuscript.

***-Ku acetylation deficient mutant (Ku70 K282A): The authors showed a reduced binding of the constitutive acetylation mimicking Ku70 mutant in comparison to wildtype Ku. Since acetylation of K282 seems to be important, a mutant deficient for acetylation should invert the effect (and bind to 5'p53) and underlining the role of K282 acetylation in Ku binding to RNA.***

As requested by the referee, to make this set of data more convincing, we performed binding experiments with an additional Ku70 mutant mimicking constitutive acetylation K282/348Q (as in [2] and with the a Ku70 mutant deficient for acetylation, i.e. K282/348R (Fig. 4G, revised version). We have also provided quantification/statistics for Fig. 4G and Supplemental Fig. 6B (revised version). To create acetylation deficient Ku70 mutants, we have introduced lysine-to-arginine (K to R) substitutions that avoid acetylation but keep positive charges, thus mimicking the non-acetylated form. Consistent with Fig. 4H (Supplemental Fig. 6B in the revised version), we now showed that the Ku70 K282/348Q mutant but not the K282/348R mutant reduced the ability of Ku to bind the p53 5' UTR in RNA pull down assays.

***Furthermore, it would be interesting to see that DNA damage does not have the same effect on this mutant (K282A) than on wildtype Ku in terms of translational regulation of p53.***

As indicated above, overexpression/rescue experiments following analysis of p53 expression from endogenous/reporter p53 mRNAs were uninterpretable due to the activation of DNA damage signaling pathways resulting from ectopic expression of Ku70/80. This was the case also after treatment with bleomycin and TSA (data not shown). One way to get around these side effects would be to test Ku70/Ku80 overexpression together with luciferase reporters under the control of the p53 5'UTR. However, as discussed above, luciferase reporters are probably not suitable to study Ku-mediated regulation of the p53 stem-loop structure.

- Experimental controls:

***A) UV cross-linking assay (Figure 2C): to support the statement "Ku binding to the 5'UTR is direct and specific" (page 7 line18) a negative control and a non-cross-linked control should be included (at least in the supplement).***

We have now included the controls suggested by the referees in Supplemental Fig. 3 (revised version).

 

***B) p53 translation /polysomal gradient: a negative control not shifting from the sub-polysomal to polysomal fraction (also here in the supplement figures) after Ku depletion would further strengthen the data (although the comparison in Fig3b is done to the corresponding Input).***

We have modified Fig. 3B (Fig. 3A in the revised version) to add a negative control (HPRT) not shifting from the sub-polysomal to polysomal fraction.

***C) Assay "p53mRNA as translational target of Ku" (Figure 3C): This assay nicely shows the effect of the B2loop in the 5'UTR on Ku mediated p53 translation. Nevertheless, loading controls and an indicated quantification of the western blot bands would be beneficial (same for Fig. 4A and 4C).***

We have now added loading controls for Fig. 3C (Fig. 3B in the revised version) and quantifications for Fig. 4A and 4C (Fig. 4B in the revised version).

***- Fig 4F: It would be great if the authors could include the difference of the two DMSO conditions. Since once the outcome is Ku acetylation and the other time not.***

There is no DMSO in Fig. 4F. If the referee meant Fig. 4D, the difference in basal Ku acetylation between the two DMSO conditions can be explained by the different western blot conditions (blocking and antibody concentration). In Fig. 4C (revised version) we now show the blot of acetylated Ku after DMSO/TSA treatment using the same western-blot conditions as for DMSO/ETO treatment.

***Consistency in writing: DNA-damage vs DNA damage, crosslinking vs cross-linking or DNA-binding domain vs DNA binding domain.***

We apologize for these inconsistencies; we have now modified the text.

**References**

1. Bombarde O, Boby C, Gomez D, Frit P, Giraud-Panis MJ, Gilson E, Salles B, Calsou P (2010) TRF2/RAP1 and DNA-PK mediate a double protection against joining at telomeric ends. *EMBO J* **29**: 1573-84
2. Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C, Frye R, Ploegh H, Kessler BM, Sinclair DA (2004) Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* **13**: 627-38

2nd Editorial Decision 08 January 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referees, and I am happy to tell you that all support the publication of your revised study.

Referee 2 only has a few minor comments which I would like you to address before we can proceed with the official acceptance of your manuscript.

Given the 5 main figures, we should publish your study as a scientific report. The character count is slightly above our limit, but we can leave it as is. Please move the entire materials and methods section to the main manuscript file, this section is not included in the character count and must be part of the main manuscript. We also do not have supplementary figures anymore, they are called expanded view (EV1, 2, etc) figures now. Can you please change all names, also in the manuscript text, and upload each EV figure as separate files? The legends for EV figures need to be added to the end of the main manuscript file. The advantage of EV figures is that they are integrated into the main manuscript online and expand when they are clicked.

Regarding statistics, Fig 2B and SF5B mention n=2, in which case no error bars can be shown. You can either show all single data points for both experiments along with their mean, or repeat the

 

experiment one more time and calculate error bars. Please also specify the error bars in the latter case, and for SF4A as well. SF1F is missing a scale bar, please add.

Please answer all questions in the author checklist under B-Statistics. Given that you calculate p values, these questions must be answered.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The authors have provided the requested quantification and have addressed all of my previous concerns. In addition, I believe that they have also made considerable effort to address all of the points raised in the previous round of review. In my opinion, the data now fully support the conclusions of this interesting study

Referee #2:

The authors have provided a strong response to the reviewers' concerns. Their revised manuscript makes a strong case for an important new role of Ku protein in regulating p53 mRNA translation through direct Ku-mRNA binding. The new Supple Fig. 4A showing EMSA analysis is a strong addition. While the paper could be published without further revision, I note:
p. 5 lines 9-10, Concomitantly, the levelS (should be plural) and it is not the levels of these genes, but rather of their protein products.
Fig. 5, the left panel might more accurately show low p53 levels (NOT total inhibition), and the right panel a modestly higher level of p53 leading to apoptosis.

Referee #3:

This manuscript describes a novel role for the DNA repair protein-complex Ku in translational regulation. As written in the first revision round, the manuscript illustrates two new important facts for the DNA damage and the translational control fields:
a) Ku is acting as an RNA binding protein in post-transcriptional regulation
b) Ku´s ability to bind to its target RNAs is regulated over acetylation.

To my point of view, all my comments were sufficiently addressed.
1) Effect of Ku overexpression: It is interesting to see that the rescue / overexpression experiments do not simply invert the effect on p53 translation, but trigger another mechanism effecting p53 protein stability (over phosphorylation) per se. Furthermore, these data do not impair the value of the descripted Ku knock-down effect.
2) Ku acetylation deficient mutant: The inclusion of this mutant indeed reinforced the author's data, that Ku70´s ability to bind to p53 5´UTR is dependent on acetylation.
3) Experimental controls: the authors added all additional demanded controls.

Taken together, I consider this manuscript as suitable for EMBO Reports.

 

2nd Revision - authors' response 25 January 2016

Thank you for the positive decision on our manuscript "A novel cytoprotective function for the DNA repair protein Ku in regulating p53 mRNA translation and function" (EMBOR-2015-401181V2). We are delighted with the overall positive comments from all referees.

As requested, we have modified the manuscript and the figure legends for Fig. 2B et EV5B, specified error bars for Fig. EV4A and added a scale bar for Fig. EV1F.

Concerning the comments of Referee 2 on Fig. 5, we modified the figure legend to make clear that the depicted model proposes that Ku contributes to the low steady-state level of p53 under normal growth conditions by suppressing p53 mRNA translation and that this inhibitory mechanism is abrogated due to damage-induced Ku acetylation, thereby allowing p53 upregulation.

We hope that our revision addresses the concerns of the referees and that the revised manuscript now fits for publication in EMBO reports.

3rd Editorial Decision 27 January 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

 

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

| |
|---|
| Corresponding Author Name: |
| Journal Submitted to: |
| Manuscript Number: |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi^2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Experiments (in vitro cell based) were performed independently 3 times (indicated n=3 in figure legends) except for the Fig1A and 4B which were performed 4 times (indicated n=4 in figure legends) and for the Fig2B and S5B which were performed 2 times (indicated n=2 in figure legends). For these latter, the histogram represents the mean and the deviation represents difference between all single data points. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | NA |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | Statistical tests are specified in the figure legend of each figures. Unpaired test was used to compare the means of two independent samples. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| Is there an estimate of variation within each group of data? | NA |
| Is the variance similar between the groups that are being statistically compared? | NA |

## C- Reagents

## USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | All the informations and references for antibodies are specified in the SUPPLEMENTAL MATERIALS AND METHODS in the section "Preparation of cell extracts and immunoblotting" |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | The source of all cell lines and the test used to verify mycoplasma contamination are specified in the MATERIALS AND METHODS in the section "Cell culture and transfections" |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

| | |
|---|---|
| 23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |