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Translation-dependent mechanisms lead to PML upregulation and mediate oncogenic K-RAS induced cellular senescence

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Transaction Report:

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

1st Editorial Decision 10 June 2011

Thank you for the submission of your manuscript "Translation-dependent mechanisms lead to PML upregulation and mediate oncogenic K-RAS induced replicative senescence" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, all reviewers highlight that the model in Fig 4F should be supported by additional data. Reviewers 1 and 2 also highlight that important controls should be included to allow for unequivocal interpretation of the data. Of special note in this regard is the assessment of senescence in Fig 4E. Importantly, reviewer 1 feels that the contribution of the PML 5'UTR to K-RAS-induced sensescence should be investigated in PML ko MEFs and reviewer 3 points out that the relevance of the observations for OIS should be further investigated.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

The paper by Pandolfi and colleagues entitled ÑTranslation-dependent mechanisms lead to PML upregulation and mediate oncogenic K-Ras-induced replicative senescence" addresses a/another mechanism by which oncogenic K-Ras induces the promyelocytic leukemia tumor suppressor gene product (PML). The authors report that K-Ras activates the MAPK-to-TSC2-mTOR-eIF4E cascade to promote PML translation even in the absence of p53. This mechanism is novel, given the current view that PML expression levels are increased in oncogene-induced senescence as a direct transcriptional target of p53.
This is a well-structured, elegantly designed and technically properly conducted piece of work on an important, and, thus far, underrecognized mechanism in OIS. Using a doxycycline-dependent, pneumocyte-targeted transgenic K-Ras-G12D mouse model, the authors show increased and p53-independent PML expression upon Ras activation. In both p53-proficient and -deficient cells PML transcript levels did not differ prior to and following Ras activation, but Ras-mediated PML protein induction was abrogated when protein biosynthesis was blocked by cycloheximide or when mTOR function was inhibited by rapamycin. The authors further demonstrate that activation of MEK, like Ras, drives PML expression in p53-deficient cells. In line with their previously reported link regarding the inhibitory action of ERK1/2 on the mTOR antagonist TSC2, a non-phoshorylatable TSC2 mutant now impaired induction of PML by oncogenic Ras. Knock-down of the mTOR-controlled translation initiation factor eIF4E reduced PML induction by Ras. A selective advantage of 5'-cap-mediated translation could only be observed upon Ras induction, and was, in turn, lost upon MEK1 or mTOR, but not PI3K inhibition, which is consistent with the postulated ERK1/2-to-TSC2 (downstream of PI3K) crosstalk. Finally, the authors detected an increased loading of PML mRNA onto heavy polysomes in the context of oncogenic Ras that was lost when rapamycin was added. Ultimately, the authors observed increased expression of PML from 5'-capped mRNA (as compared to non-capped transcripts) in response to Ras, and reported a correlation with a higher frequency of SA-b-gal-positive cells.
The experimental evidence is clearly presented, and data are explained in a crisp and logical way in the text. The discussion, although not covering all prominent and potentially conflicting findings in the field (see below), does not miss to point towards potentially important clinical implications - which is a particular merit of the paper and relevant for the journal chosen.
The following points should be addressed before publication in EMBO MolMed might be considered:
1. While conclusively presented, the data challenge previously published evidence, which should be discussed to ensure a balanced view of the signaling processes studied. Peter Adams and colleagues recently reported that an activated PI3K/Akt pathway is able to counteract Ras-induced senescence, although acknowledging that PI3K/Akt on its own may promote senescence (Kennedy-AL et al, Cancer Cell, 2011). Likewise, Karen Cichowski and colleagues presented evidence that Ras-induced senescence, after an initial activation of PI3K/Akt signaling, actually depends on the negative feedback regulation that eventually results in Akt inactivation and FoxO reactivation (Courtois-Cox-S et al, Cancer Cell, 2006). Hence, Adams finds activated Akt to bypass Ras-induced senescence - although it should actually contribute to PML induction via enforcing TSC2 inhibition. And Cichowski sees Akt activity reduced in Ras-induced senescence, which, in turn, should reactivate TSC2, and, thus, ultimately result in lower PML expression levels.
2. The authors themselves provided evidence in previous publications that PTEN loss induces cellular senescence ("PICS") and suggested enhanced translation of p53 as the underlying mechanism (Almonti-A et al, JCI, 2010; and also Chen-Z et al, Nature, 2005). So, what are the relative roles of p53 and PML in OIS and PICS?
3. In light of the previous points, the paper should be experimentally sharpened regarding the role of p53 and PML in cellular senescence. The PML expression/OIS experiment in Figure 4E was presumably carried out in p53-proficient MEFs. Since p53-/- MEFs do not senesce in response to

oncogenic Ras, it would be informative to see the impact of a transfection-based 5'-cap PML mRNA on the chosen readout, i.e. the frequency of SA-b-gal-positive cells in p53-/- MEFs.
What's really missing is to test senescence induction following 5'-cap-UTR vs. non-capped PML plasmid transfection into PML-/- MEFs {plus minus} oncogenic Ras. Since PML-/- MEFs fail to senesce in response to oncogenic Ras (the authors' own data; Pearson-M et al, Nature, 2000), only this experiment will give insights about the physiological role of PML cap translation in Ras-induced senescence.
At what time were cells scored for SA-b-gal reactivity in Fig. 4E? If these are wt MEFs, around 15% "blue cells" would be extremely low in response to Ras (known to produce 80 or more percent of SA-b-gal-positive cells at 5-7 days after Ras infection) - and could not be considered a senescent cell population. Therefore, additional BrdU/PI and growth curve data should be provided here.

Minor issue:
The manuscript would benefit from cross-reading by a native speaker-scientist

Referee #2:

The manuscript by Scaglioni et al studies the regulation of PML by K-Ras. They show that PML translation is regulated by the Ras-Mek-mTOR cascade and suggest that this effect could be relevant for oncogene-induced senescence. A role for K-Ras in control of PML translation is potentially interesting. However, the data in the manuscript are too preliminary to support the conclusions and additional experiments and controls are necessary.

Specific points

1. Except for Figure 1 and 4E, the study is exclusively performed in p53-null cells. Controls with wt MEFS should be included at relevant points throughout the manuscript (for instance Figures 2A or 4D) to help interpret the results.

2. A previous report showed that the induction of PML RNA and protein by H-Ras is lost in p53-null MEFs (de Stanchina, Mol Cell, 2004). However, Figure 1C of this manuscript shows normal PML protein induction in p53-null MEFs. How do the authors explain this apparent contradiction? One potential factor is the use of K-Ras versus H-Ras. Have the authors tested if there is any difference between K-Ras and H-Ras in this context?

3. It is not obvious why the data in Figure 2C supports the conclusion that PML regulation is predominantly posttranscriptional in these cells. One would expect that CHX would block translation of any given protein, even if it is also transcriptionally regulated. The effect of rapamycin is out of place here, it should be moved to Figure 3.

4. Figure 4B shows results based on transient transfection. There is a concern that differences in transfection efficiency can distort the results (for instance, the authors point out that "p53 null MEFs expressing onc-K-RAS are transfected less efficiently than p53 null MEFs transduced with babe"). The authors should include controls for transfection efficiency. Also, it would be useful to measure transcript levels for each ectopic construct, to confirm that the effect is translational, as they suggest. The EGFP signal in BabePuro cells is too intense (probably saturated), a shorter exposure should be shown to allow for better quantitation and comparison with RAS-infected cells. The problem of efficiency due to transient transfection could also be circumvented by the use of retroviral serial infections, as in panel 4E.

5. In 4C, data with BabePuro should also be included, to show if this a general effect in translation mediated by mTOR or specific to Ras-induced upregulation of PML.

6. Fig 4D convincingly shows loading of PML transcripts in polysomes in the Tet-Ras p53KO MEFS. This is the most compelling data showing a specific role in translation. However, the data could be improved with additional controls. Positive and negative control genes (i.e., loaded into polysomes or not) could be used, and the same experiment in wild-type MEFs should be shown.

7. In Figure 4E, are the MEFS wild-type? This fact is not specified in the text or the figure legend.

The conclusion that PML translation is relevant for OIS is mostly based on this figure. However, the data do not seem strong enough. The authors use the small difference between the LNC-5'UTR-PML and LNC-PML signals in Babe-RAS cells to conclude that the UTR contributes to PML upregulation by Ras. The differences are marginal and, with the same logic, one could also conclude that the UTR has the opposite effect in Babe-Puro-infected cells. Ectopic expression of PML has been shown to induce senescence. In this experiment, did PML alone induce senescence? If so, did the inclusion of the 5'UTR have any effect? In this panel, senescence is measured by SABetaGal staining. The graph shows only minor differences whose statistical significance is not clear (The p values seem incorrect. Is p higher or lower than 0.05?) To obtain more conclusive data, the authors should use additional senescence readouts like proliferation rate or p19Arf induction.

8. The model in Figure 4F suggests that p53-dependent transcriptional regulation and p53-independent translational control are two alternative pathways for PML regulation by Ras. The implication would be that both should be in place in p53-positive fibroblasts. Is there any evidence for the existence of both mechanisms in wild type cells?

9. The major claim of the manuscript is that the regulation of PML translation by K-Ras is important in oncogene-induced senescence. However, the relevance for OIS is not sufficiently supported by the data. Induction in adenomas points at a link to senescence in vivo, but the role of translation in that setting is not known. On the other hand, most of the cellular analysis is done in p53-deficient MEFs, where K-Ras should not induce senescence. If the authors' model is correct, the effect on translation would be predominant in p53 null MEFs, showing an inverse correlation with induction of senescence. In this regard, it would be interesting to see if these observations can be extended to human fibroblasts, where OIS is not strictly p53-dependent.

Referee #3 (Comments on Novelty/Model System):

The quality of the experiments is high in general. The idea that PML can be controlled at the translastional levels by Ras and not just through transcriptional p53 control is novel. model system is appropriate but how this is going to impact into medicine is very preliminar at the moment. At this stage this is a basic science (interesting) report. My major caveat is that many of the results are too subtle to be able to evaluate how much this mechanism really impacts in PML regulation by Ras

Referee #3 (Other Remarks):

The manuscript by Scaglioni and collaborators identifies a novel mechanism behind the regulation of PML by Ras that is p53-independent and relays on eIF4E dependent translation regulation.

The idea is novel and interesting, although mainly from a basic science point of view. Experiments are well executed, but some of the effects seems to subtle. Some of the PML western quantificatons (for example Fig1E, F, 2E,) are really showing minimal effects what really raises the question of what is the relevance of this particular mechanism compaed with the p53-dependent transcriptional induction.

Particularly, the only 'functional' experiment is the SA-beta gal quantification of the cooperation on senescence between Ras and PML (in the presence or absence of the 5'UTR element). Again the effects are very subtle and the overall percentage of senescence cells is minimal. a better functional proof, in anyway of the relevance of this mechanism when compared with p53-control of PML transcription should be presented.

1st Revision - Authors' Response 15 September 2011

First of all, we would like to thank the reviewers for providing constructive comments. In our opinion, we have been able to successfully address each of the points raised, significantly strengthening the manuscript. Specifically, we present several new data providing the controls to the experiments presented in Fig. 2 and 4.

Therefore, the findings presented in this amended manuscript further support the notion that oncogenic K-RAS upregulates PML through translational mechanisms.

Reviewer #1. We thank the reviewer for recognizing " that this is a well-structured, elegantly designed and technically properly conducted piece of work on an important and thus far, underrecognized mechanisms of OIS". This reviewer raised several comments, which we have addressed as follows:

Comment #1. As suggested, we have added in the discussion section of this manuscript the work published previously by Kennedy et al. and Courtois-Cox et al. This information is now discussed on page 11.

Comment #2. As suggested, we have discussed our findings in relation with the report of Alimonti et al., from our group. We described that PTEN deficiency promotes PTEN induced cellular senescence (PICS) by upregulating p53 through a translational, mTOR dependent mechanism. PML regulates P53 by promoting its acetylation, therefore we think that it is likely that activation of the PI3K signaling pathway results in the co-ordinated upregulation of tumor suppressors to counteract deregulated cellular proliferation. This information is now discussed on page 11.

Comment #3. The reviewer requested several clarifications:

i) he/she asked about the p53 status of the cells used in Fig. 4E. We have now edited the paper to indicate that these experiments were performed with IMR90 human fibroblasts.

ii) The reviewer also suggested to express the 5' cap-PML mRNA in p53 null cells to assess its impact on the induction of OIS markers. We agree that this experiment would be useful to assess the functional role of the PML 5' UTR sequence, however, this experiment cannot be done because PML is unable to induce OIS in p53 deficient cells.

iii) The reviewer asked to express the 5' cap-PML mRNA in *Pml* null MEFs to assess its impact on OIS. Multiple PML isoforms coexist in the cell. Replicative senescence is not reconstituted in Pml null MEFS by transfection of a single PML isoform (Bichof et al, EMBO Journal, 2002), therefore this experiment is not feasible. Instead, transfection of PML isoform IV induces OIS in PML competent human fibroblasts. For this reason we performed these experiments in IMR90 and BJ human fibroblasts.

iv) We apologize for not explain that this experiment was performed in human fibroblasts. As suggested, we performed proliferation assays to assess OIS in IMR90 cells. We have also performed beta gal assays in BJ human fibroblasts (now shown in supplemental Fig. 1D). We agree that the percentage of beta gal positive cells is somewhat low. However, we find that BJ cells are relatively resistant to OIS induction. Using cells that undergo beta gal staining in percentages higher than 80%, such as IMR90 or WI38, would preclude the execution of these experiments. Moreover, we have strictly counted only intense nuclear beta gal staining. We presented the result of the growth curves in Fig. 4E and a histogram showing beta gal positive cells in supplemental Fig. 1C.

Reviewer #2. We thank this reviewer for raising several comments that improved our manuscript.

Comment #1. As suggested, we have performed the experiments shown in figures 2A and 4 D with p53 competent cells. We now provided the results of these experiments in Fig. 2C, Fig. Supplemental Fig. 1A and D of this amended manuscript. We determined that Pml mRNA is modestly increased by oncogenic K-RAS in wild type MEFs and that PML mRNA is uploaded on polysomes also in p53 competent cells.

Comment #2. We agree with the reviewer that our data are in apparent contradiction with the previous report of de Stanchina et al. showing that H-RAS induces Pml upregulation through a transcriptional mechanism that depends on p53. As requested, we determined whether H-RAS induces Pml upregulation in wild type MEFS. We discovered that also H-RAS upregulates Pml in p53 null MEFS (now reported in page 6 and supplementary Figure 1). We can only speculate about the reasons of this discrepancy. As demonstrated in answer #1, Pml mRNA is modestly upregulated by K-RAS and H-RAS in p53 competent MEFS. Therefore, it is possible that Pml is also controlled at the transcriptional level and that the differences between the study of de Stanchina are due to differences in experimental conditions.

Comment #3. We agree with the reviewer that Fig. 2C of the initial manuscript does not conclusively prove that PML is regulated primarily by translation, therefore we omitted it in this amended manuscript.

Comment #4. The reviewer expressed concerns regarding differences in transfection efficiency of the experiment shown in Fig. 4B. This experiment relies on the transfection of several plasmids, thus we agree that this is a valid concern. We apologize for not mentioning in the initial manuscript that we controlled for efficiency of transfection by visually counting the percentage of GFP positive cells and by monitoring luciferase expression from an input plasmid. We agree with the suggestion to use cells serially transduced with retroviruses. In fact, we present the results of such experiment in Fig. 4E. We have mentioned these facts in Pag. 8 of the revised manuscript.

Comment #5. As suggested, we have included data of cells transduced with babe puro after treatment with rapamycin, LY294002 or UO129. These data demonstrate that the effects seen are specific for cells expressing oncogenic K-RAS (Fig. 4C).

Comment #6. The reviewer asked to perform polysome fractionation experiments to assess PML mRNA loading on polysomes. We show the results of these experiments in supplemental figure 1.

Comment 7. As requested we have performed proliferation assays to complement the results shown in Fig. 4E. These results confirm that the PML 5' UTR upregulates PML in oncogenic K-RAS expressing cells. We have also clarified in the figure legend and text, that these experiments were performed in IMR90 and BJ cells (human immortalized fibroblasts). We have also corrected the typing error noted in the histogram now shown in supplemental Fig. 1.

Comment 8. We have added text to propose that transcription and translational mechanisms are responsible for PML upregulation in oncogenic K-RAS expressing cells (Pag. 10).

Comment 9. We apologize for not stressing in the manuscript initially submitted that the experiments shown in panel 4E were performed in IMR90 cells. Therefore, we propose that our observations are valid in both murine and human fibroblasts.

Reviewer #3.

This reviewer asked about the relevance of translational regulation of PML in determining oncogenic K-RAS OIS. We present this data in Fig. 4E. We also think that the magnitude of the phenotypes we have observed through the manuscript in is consistent with the literature.

Reviewer 3 (as reviewer 2) also suggested to perform a functional assay to complement the experiments shown in panel 4E of the initial manuscript. We now present a proliferation assays in Fig. 4E (as discussed to the comments to reviewer #2)

2nd Editorial Decision 18 October 2011

Thank you for the submission of your revised manuscript " Translation-dependent mechanisms lead to PML upregulation and mediate oncogenic K-RAS induced replicative senescence" to EMBO Molecular Medicine. We have now received the reports from the reviewers who were asked to re-review your manuscript.

You will see that the referees remain interested in the study but referees 1 and 2 still raise a number of significant concerns about the conclusiveness of the results. However, we would be willing to consider a revised manuscript with the understanding that the referee concerns must be convincingly and conclusively addressed.

Importantly, reviewer #2 remains largely unconvinced concerning the mechanistic evidence directly linking the regulation of PML translation with induction of senescence. However, he/she suggests an experiment to potentially provide some mechanistic evidence. This reviewer also highlights issues concerning the use of cells derived from different species.

We also note that reviewer #1 feels that the experiment regarding the physiological role of PML cap translation in Ras-induced senescence should be performed.

On a more editorial note, please see below for information regarding EMBO Molecular Medicine guidelines for statistical analysis of data (p value calculation is mentioned in the Material and Methods but no values are mentioned in the text or figure legends), formatting of supplementary information and the mention of 'unpublished data' (this would refer specifically to the estimation of transfection efficiency).

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

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

This is now the revised version of the paper entitled ÑTranslation-dependent mechanisms lead to PML upregulation and mediate oncogenic K-Ras-induced replicative senescence" by Pier Paolo Pandolfi and colleagues.

As previously stated, the overall impression of the current manuscript remains positive, and the revision clearly is an improvement with respect to concerns raised by all referees.

However, I cannot find all - including particularly relevant - comments being addressed satisfyingly. For simplicity, I refer to the "answers to reviewer #1" as listed in the point-by-point rebuttal:

Comment #1: Yes, the authors have now included a paragraph in the discussion (page 11/12) where they briefly touch on the contradictory findings published by the Cichowski and the Adams laboratories. While it might not be easy for the reader to catch the scope of these conflicting results as described in the discussion, there is no interpretation or integration of the apparently incompatible findings. So far, the authors, as they state earlier in this paragraph, view this problem as "context-dependent signaling pathways and feedback mechanisms".

Comment #2: In the same paragraph, the authors refer to their own previous finding on "PTEN loss-induced cellular senescence (PICS)", where the wording should be amended ("...PTEN loss,... ..., induces PTEN-induced cellular senescence [PICS]...").
Comment #3-i: OK.
Comment #3-ii: Given the role of PML to operate up- and downstream of p53 (work mostly by the authors and by de Stanchina et al., Mol. Cell, 2004), it would have been informative and more clear to the reader of this manuscript to actually demonstrate that indeed, despite p53-independent upregulation of PML, PML is unable to mediate senescence (directly and in response to oncogenic Ras) in p53-deficient cells.
Comment #3-iii: I guess I did not ask to express 5' cap-PML mRNA in PML-deficient cells in the absence of Ras (as done by Bischof et al., EMBO J., 2002 - and not leading to senescence), but I suggested to compare senescence induction in 5' capped vs. non-capped PML in PML-deficient cells {plus minus} oncogenic Ras, i.e. obviously in the presence of activated Ras. This is a key experiment - and certainly a feasible one.
Comment #3-iv: OK. Just a side note: I hope the authors have not "strictly counted only intense nuclear beta gal staining" as they stated here - since SA-b-gal staining is restricted to the peri-nuclear cytoplasm.

Referee #2:

The revised version of the manuscript includes additional data and controls in response to the reviewers' comments that have improved the technical quality of some of the data. However, despite these improvements, the evidence linking K-Ras regulation of PML translation to senescence remains weak and thus, the functional relevance of the data for oncogene-induced senescence is not sufficiently demonstrated.

Oddly, the authors have chosen to use human fibroblasts for the functional assays, even though the rest of the data comes from mouse fibroblasts. It is well established that there are important differences between human and mouse cells in terms of senescence. Ideally, functional data with mouse fibroblasts should be provided. If human cells are used, the authors need to show that the main observations from mouse fibroblasts can be extended to human cells. For instance, is endogenous PML protein upregulated during OIS in human fibroblasts? The new data in Figure 4E shows that PML modestly potentiates K-Ras-induced senescence, and this effect is stronger when the 5'UTR is present. Ras is a potent senescence inductor by itself in this cell type. As suggested in the original comments, a more direct and sensitive assay would be to test the ability of ectopic PML (with or without UTR) to trigger senescence. The proliferation and SABetaGal data shown were obtained from two different strains of human fibroblasts, both assays should be shown for the same strain.

An additional issue that remains to be clarified is whether the effect of K-Ras on PML is specific to senescence or rather is linked to Ras-signaling irrespective of cellular outcome. The fact that K-Ras regulates PML translation in p53-deficent MEFs, that do not undergo senescence, already indicates that there is not a strict correlation. This notion could also be tested with experiments in human cells in conditions of senescence bypass (for instance, co-expressing oncogenic Ras and E1a).

Other points:

The authors describe the IMR90 and BJ cells used as "immortalized fibroblasts". They should clarify what they mean. Did they use early passage, primary fibroblasts or were they immortalized by any means?

The new controls for the polysome fractionation experiments in figure 4D are performed in BJ human fibroblasts (Supp. Fig 1C), while the existing data came from p53-deficient mouse fibroblasts. A complete set of data from the same cell type should be shown.

The concerns about transfection efficiency in Figure 4 have not been addressed. Considering the small differences shown, this issue is important to prove the validity of their conclusions. The authors mention that they have controlled for efficiency of transfection, but the actual data showing comparable efficiencies is not shown. They should show some transfection control, or use

alternative methods where efficiency is not an issue. The same loading control should be used in panels 4B, 4C and 4E.

The term "replicative senescence" is commonly used to refer to senescence associated to accumulation of population doublings, not oncogene activation.

Referee #3 (Comments on Novelty/Model System):

No problems. I think the quality of experiments and the choice of the system is adequate.

Referee #3 (Other Remarks):

The revised version of the manuscript has improved and this reviewer is satisfied withe the reply of the authors.

2nd Revision - Authors' Response 11 February 2012

First of all, we would like to thank the reviewers for taking the time to review our manuscript. The reviewers expressed several remaining concerns. We have addressed each one of them with several new experiments.

In our view, our new data provide further support to the conclusion that not only oncogenic K-RAS upregulates PML through translational mechanisms, but that this event is biologically significant.

We will discuss our findings and conclusions below.

Reviewer #1. The reviewer expressed that he/she had a “positive view of the manuscript” and appreciated the “the current version of the manuscript was clearly an improvement”. However, he/she had several remaining concerns, which we will discuss below.

Comment #1. The reviewer asked us to discuss papers senior authored by Dr. Karen Cichowski and Peter Adams, which are not coherent with each other. We have not attempted to replicate in our laboratories these experiments, therefore we do not have a first-hand opinion about these findings. We are not prepared to comment further the results of others. In our opinion, these conflicting results underscore the fact that replicative senescence is most likely governed by complex, context dependent mechanisms that are only beginning to emerge. Thus, our report that PML is regulated by post-translational mechanisms, adds a novel mechanistic insight into the networks that regulate replicative senescence.

Comment #2. As suggested, we have edited the sentence:…PTEN loss,…, induces PTEN-induced cellular senescence” (Pag. 12).

Comment #3. The reviewer asked to: “demonstrate that, indeed, despite p53-independent upregulation of PML, PML is unable to mediate senescence in p53 deficient cells”.

It is firmly established that p53 is essential for the induction of oncogene induced replicative senescence (OIS) (1). Consequently, PML requires p53 to exert its prosenescence function (2, 3). Therefore, it is expected that PML would be unable to induce OIS (i.e. a permanent cell cycle arrest) in p53 null MEFs. Nevertheless, we have performed the experiment requested by the reviewer.

We determined that expression of a PML cDNA devoid of the PML 5’ UTR sequence, did not appreciably affect the ability of oncogenic K-RAS to promote cell proliferation in p53 null

MEFS. In addition, we found that expression of a PML cDNA comprising its 5'UTR, modestly suppressed cellular proliferation of p53 null MEFs expressing oncogenic K-RAS. Finally, in the absence of oncogenic K-RAS, the presence of the 5' UTR did not appreciably change the effects of ectopic expression of PML on cellular proliferation. We have added these data in Supplemental Fig. 2E and pag. 10.

The reviewer also asked to test the effect of the PML 5' UTR on OIS induced by oncogenic K-RAS in *Pml* null MEFs. We have performed this experiment, however we have found that expression of PML, irrespectively of the presence of the 5' UTR, did not affect oncogenic K-RAS induced replicative senescence. We have mentioned this experiment in page 10, but did not show the results because not informative.

These results are in complete agreement with a manuscript senior authored by Dr. Anne Dejean (4). This group has reported that: 1. only PML isoform IV (the one used in our studies) is able to induce OIS and 2. PML isoform IV induces OIS only in cells with a functionally intact PML gene. Thus, none of the PML isoforms is capable to induce OIS in *Pml* null MEFs (4). We replicated these experiments more that a decade ago. Taken collectively, these results indicate that PML IV is devoid of prosenescence activity in *Pml* null MEFs. For these reasons, the PML field has used immortalized diploid human fibroblasts (as we have done here) to study PML dependent OIS (2-6).

Comment #4. We edited the text to indicate that we counted perinuclear SA-b-gal (Legend Supplementary Fig. 2).

Reviewer #2.

Comment #1. The reviewer expressed the concern that human and mouse fibroblasts may not share PML dependent OIS programs. We agree that the mechanisms underlying OIS induction in human and murine cells are not identical (i.e. the Rb pathway and the regulation of telomere length have a more prominent role in human cells). However, it has been firmly established that oncogenic K-RAS induces OIS in human immortalized fibroblasts. In culture, this process depends largely on the p53 signaling network, which is shared between murine and human cells. Indeed, the seminal paper of Serrano et al. that demonstrated that oncogenic K-RAS induces OIS, described findings obtained in WI38 and IMR90 human fibroblasts (1). Moreover, it has been conclusively established by several independent laboratories that PML dependent OIS is p53 dependent and that the pathways involved in its regulation are shared between murine and human fibroblasts (see also answer to comment #3 of reviewer #1) For example, the initial experiments demonstrating that PML induces OIS were obtained in MEFS and fully reproduced in WI38 and IMR90 human fibroblasts (2, 3). Moreover it was shown that E1A bypasses the effect of PML on OIS (3)

Precisely for these reasons, and consistently with the cellular systems presently used to study OIS, we tested the effect of the PML UTR on OIS induction in IMR90 and BJ human fibroblasts. Thus, we feel that this choice of cell lines is appropriate since PML induces OIS with comparable efficiency in human and mouse fibroblasts. This choice of experimental system also underscores the importance of our findings for human disease.

Nevertheless, we followed the suggestions of the reviewer and demonstrated that, as in MEFs, PML is upregulated by oncogenic K-RAS also in human fibroblasts (Supplemental Fig. 1B-D and pag. 5).

Comment #3. As requested we present SA-b-Gal staining of IMR90 cells (Supplementary Fig. 2D).

Comment #4. As suggested, we determined that oncogenic K-RAS upregulates PML in human fibroblasts in conditions of OIS bypass (i.e. coexpressing oncogenic K-RAS and E1A) (Supplemental Fig. 1E, pag. 5).

Other points:

1. The reviewer asked a clarification regarding the nature of the IMR90 and BJ fibroblasts.

These are well-characterized human diploid fibroblasts available through the ATCC. IMR90 cells were derived by serial passage of primary fibroblasts obtained from the lungs of a 16-week female fetus (7). The BJ cell line was established by serial passage from skin taken from normal foreskin (8). Both cell lines when received from the ATCC are reported to have the capacity to attain more than 50 population doublings. We have now described the characteristic of IMR90 and BJ cells in the Material and Methods section. We have also clarified that we have used cells that underwent 10-20 passages after receiving them from the ATCC.

2. The reviewer suggested performing polysome fractionations in MEFs. These experiments require large amount of cells. For this reason they are typically performed with cell lines. It is difficult to obtain large quantities of MEFS. Since the mechanisms underlying PML dependent OIS induction in mouse and human fibroblasts are conserved (please, refer to our answer to comment #1, refer also to pag. 5 and Supplemental Fig. 1B-D), we used BJ fibroblasts. These cells grow well in culture and induce PML upon oncogenic K-RAS expression (Supplemental Fig. 1B-D and pag. 5). For these reasons we think that it is not necessary to repeat these experiments in MEFs.

3. We repeated the experiment presented in Fig. 4B. We presented the requested control of transfection efficiency in Supplemental Fig. 2B.

4. As requested, we have edited the term “replicative senescence” into “cellular senescence“ throughout the manuscript (including the title). Note that now the abbreviation OIS refers to “oncogene induced cellular senescence”.

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