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## **Oxidative stress induces an ATM-independent senescence pathway through p38 MAPK-mediated lamin B1 accumulation**

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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

20 April 2011

We have now received all three referee reports on your lamin B1-senescence manuscript submitted to The EMBO Journal. As you will see, the referees consider your findings potentially interesting but at the same time raise a significant number of substantive points, relating mainly to the conclusiveness of the current data but also to more general conceptual points. On close consideration of these points, I feel that some of them may exceed the scope of the current already quite comprehensive study, but that there are nevertheless several key points that would need to be satisfactorily addressed to warrant publication in The EMBO Journal. My conclusion is therefore that we should in principle be able to consider a substantially revised manuscript further for publication; however given the significant amount of further work and time probably required for these revisions, we would also understand if you were to rather decide to publish the study rapidly and without major changes elsewhere.

In case you decide to revise the manuscript for The EMBO Journal, we would in this case be more than happy to extend the usual three months revision time frame to up to six months upon your request. Furthermore, the key points for a successful revision in this case would be the following:

- significantly improving the assessment and scoring of nuclear shape changes - this is a key foundation of the manuscript and I feel that especially the experts from the nuclear lamina side will need to be convinced here [see referee 1's first 'specific comment', referee 2's point 4 (including useful scoring suggestions) and referee 3's point 7]
- adding at least some mechanistic insight into the mechanism of lamin B1 stabilization by p38, such as p38-mediated phosphorylation or proteasome/lysosome inhibition experiments [referee 2 point 10, referee 3 point 4]

- adding missing experiments to bolster the causality (rather than simply correlation) in some instances [referee 3 point 6: do ROS still cause senescence in absence of lamin B1; referee 2 point 7: does lamin B1 loss in A-T cells cause the same rescue effects as p38 inhibition or ROS scavenging]
- related to this is the issue of generalization: confirm that p38 inhibition or lamin B1 loss rescue senescence induced by ROS treatment or ATM inhibition also in wild-type cells [referee 1's first major point] and test if lamin B1 overexpression and nuclear alterations would also occur in at least one other form of ROS- and p38-involving senescence, such as Ras-induced senescence [referee 2 point 16]

On the other hand, points that may be beyond the scope of the current analysis and where addressing them in writing may be sufficient (nevertheless, this will require diligent discussions of these issues), are the following:

- ref 2 point 6 (if difficult to do in your transfection settings)
- ref 2 point 17 (but any relevant control data you may have to answer this would be helpful)
- ref 3 point 1

For all the remaining specific experimental and presentational questions, please try to address them as carefully and thoroughly as possible, where necessary through addition of further control data; as it is EMBO Journal policy to allow only a single round of major revision, and acceptance thus depends on the completeness of author responses in this revised version. When preparing a letter of response to the referees' comments, please bear in mind that this will form part of the 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>).

During our standard revision time (but also if an extension has been granted by the editor), competing manuscripts published elsewhere will by editorial policy 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. Please also let us know in advance whether you would require an extension of the revision time, or whether you decide against resubmitting a revised version. Of course, I'll also be happy to answer any other question you may have regarding the reports and this decision!

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

Yours sincerely,

Editor  
The EMBO Journal

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REFeree REPORTS:

Referee #1 (Remarks to the Author):

In this study the authors investigate the role of lamin B1 in stress induced senescence. Both, cells from AT patients and wild type cells exposed to oxidative stress or ATM inhibitors upregulate lamin B1 protein levels associated with nuclear shape abnormalities. Lamin B1 overexpression caused similar nuclear abnormalities and senescence. In contrast, treatment of AT cells with anti-oxidative agents caused lamin B1 downregulation and rescued nuclear morphology.

Stress induced lamin B1 increase was mediated by increased protein stability, likely mediated by p38 kinase activity. Likewise p38 kinase activators in wt cells increased, while p38 inhibitors in AT cells decreased lamin B1 levels. Similarly RNAi-mediated downregulation of p38 rescued lamin B1 levels, nuclear shape and morphology in AT cells.

Although the topic is very interesting the manuscript does not provide clear mechanistic insights in the link between oxidative stress, p38 signaling, lamin B1 upregulation and senescence. It is also not clear whether these are direct effects or just correlations. The authors compared rescue

experiments in AT cells with stress-induced effects in wt cells. One critical experiment is to test whether e.g. H2O2- or ATM inhibition-mediated senescence in wt cells can be rescued by p38 downregulation/inhibition or lamin B1 downregulation.

Furthermore, to show that lamin B1 overexpression is indeed downstream of oxidative stress, DNA damage and p38 kinase it is important to show that lamin B1 overexpression does not affect these parameters.

Specific comments:

Immunofluorescence microscopy is of poor quality and nuclear shape changes shown in Fig. 2 and 4 are not convincing.

Fig. 1: Is this effect specific for lamin B1 or are other lamins affected as well. Blots for lamin B2 and lamins A/C should be provided.

Fig. 5: A control should be added showing degradation of unrelated proteins in AT cells. I am surprised that actin is not turned over at all in 9h.

Fig. 5B: Activation or inhibition of p38 / MAPK signaling should be demonstrated.

Referee #2 (Remarks to the Author):

Comments for transmission to the authors

In this manuscript, Barascu et al present data to show that lamin B1 accumulates in cells lacking functional ATM. This lamin B1 accumulation is sufficient to induce cellular senescence, as evidenced by beta-gal staining, heterochromatic foci, and misshapen nuclei. The authors demonstrate that this lamin B1 increase is dependent on oxidative stress, which activates p38, which in turn stabilizes the protein level of lamin B1. The authors conclude that their data connect the two classes of progeroid syndromes (laminopathies and DDR defect syndromes). Research into cell cycle regulation and gene expression regulation by the nuclear periphery is an area of increasing interest - the authors' results are mostly novel, and if they prove to be generally applicable, they would attract a wide readership, but the paper would benefit if the authors expanded their findings into multiple forms of senescence or provide additional mechanism (mechanism is particularly desirable given the unexpected connection between p38 and lamin B1 protein stability). Based on the authors' data, lamin B1 should accumulate in any type of senescence with increased oxidative stress and p38 signaling, which includes replicative senescence, oncogene-induced senescence, and direct DNA damage-induced senescence. Additionally, there are several control experiments that would significantly increase the reliability of the conclusions, as I enumerate below.

Introduction: In general, this is a good introduction to senescence and lamin biology and adequately covers the appropriate literature. A few criticisms:

1) There are more grammatical and syntactical errors in the Introduction than in following sections. I suggest that the authors have a native English speaker perform a line-by-line edit of at least this section.

2) "...senescence is responsible for organism aging...": While this is a hypothesis that is prevalent in the field of senescence, it is far from being proven or even directly supported. At best, the evidence is correlative. I suggest toning down the language and stating that this is a hypothesis.

3) While there is not a lot of research into lamin B1 out there, there are several studies examining lamin B1 loss, and these data should be discussed, as it seems that lamin B1 loss phenocopies many of the effects the authors see with lamin B1 accumulation, which is surprising. For example, here are a few papers that suggest that loss of lamin B1 induces premature senescence and misshapen nuclei:  
- Lammerding J, et al., (2006) J Biol Chem 281: 25768-25780  
- Vergnes L, et al., (2004) Proc Natl Acad Sci U S A 101: 10428-10433  
These papers do not conflict with the authors' data directly; however it is surprising that both the accumulation and the depletion of a protein induce similar phenotypes.

Figure 1: good: the authors examine multiple cell strains and tissue types. I have no critiques

Figure 2:

4) How was nuclear morphology assessed? If this was done by eye, it strikes me as highly

subjective. An example of objective quantitation would be to use an image analysis software like CellProfiler ([cellprofiler.org](http://cellprofiler.org)) to analyze the negative curvature of the nucleus, determine a curvature score, and set a threshold to define normal vs abnormal (or even better - compare the two histograms directly rather than making a binary distinction). This analysis could be performed on existing images - no new experiments necessary. At the same time, it would be interesting to quantitate lamin B1 level by IF and determine if there is a correlation between lamin B1 level and nuclear curvature/abnormality, as one would predict from the authors' data.

5) A western blot showing the level of lamin B1 overexpression would be useful here as a supplement, as would a western blot showing the level of lamin A/C in wild-type versus A-T cells, because the authors are claiming a role for lamin B1 specifically rather than a role for lamins in general. It is worth noting that the difference in lamin B1 level between control and lamin B1 plasmid (b) looks no different than the difference in lamin A/C between wild-type and A-T cells (a) to my eye. Additionally, why do the authors use lamin A/C in (a) but lamin B1 in (b) - this inconsistency is needlessly confusing.

Figure 3:

6) Beta-gal staining and SAHF formation are both used as markers of senescence, but both have been shown to occur in the absence of growth arrest. It would be fairly quick to perform a 1-2 week growth curve or colony formation assay on lamin B1 overexpressing cells to determine whether they were actually growth arrested, and thus senescent.

7) As lamin B1 overexpression in normal cells induces beta-gal and aberrant nuclear shape, it would provide symmetry to test whether lamin B1 depletion in A-T cells delays beta-gal staining and reduces aberrant nuclear shape. Additionally, this would support the subsequent conclusions that NAC and SB203580, which reduce beta-gal and aberrant nuclear morphology (this is well known), accomplish this delay by decreasing lamin B1. This would also address the seeming discrepancy with previously published literature, as I discussed in point #3.

Figure 4: mostly good. I appreciate that the authors looked at both cell types and multiple inducers of ROS.

8) A minor point is that the lamin B1 levels in NAC-treated A-T cells look higher than the lamin B1 levels of untreated A-T cells in (c) - in fact, they look as high as the lamin B1 levels of H<sub>2</sub>O<sub>2</sub> treated wild-type cells. While IF is not particularly quantitative, the trend certainly shouldn't be opposite what is seen with the western data in (a).

Figure 5:

9) Using cycloheximide to prevent protein translation and determine protein stability is not uncommon; however, the results must be interpreted with caution. Cycloheximide is a potent inducer of apoptosis (e.g. Blom WM, et al., 1999. *Biochem Pharmacol* 58: 1891-1898). The concentration and time is cell-type dependent, but 3-9 hours at 50 ug/mL is well within the range of many published studies. Apoptosis, of course, is characterized by lamin B1 cleavage and degradation (e.g. Kivinen K et al., 2005. *Exp Cell Res* 311: 62-73). Because A-T cells are resistant to apoptosis (e.g. 2nd paragraph of Cosentino et al., 2011 in the author's bibliography), it is conceivable that the difference in protein stability seen between A-T cells and wild-type cells is simply because A-T cells are more resistant to cycloheximide-induced apoptosis and thus lamin B1 degradation. To test this possibility, the authors could repeat the experiment in the presence of Z-VAD, which is a caspase-3 inhibitor. If Lamin B1 is still decreased in the presence of cycloheximide, it suggests a true stability shift.

10) I don't understand why the authors state that p38MAPK seemed like a good candidate for lamin B1 regulation (page 9, bottom), given the change in protein stability. To my knowledge there are zero published studies connecting lamin B1 regulation to p38MAPK, and p38MAPK is not generally reported to affect protein stability - it primarily regulates mRNA stability and transcription. Some explanation of how the authors think p38MAPK can affect protein stability at all, let alone lamin B1 stability, would be useful. It would also be useful to include any senescence regulators that the authors found not to affect lamin B1 (e.g. ERK, JNK, NF-kB, or p53, etc), though potentially outside the scope of the manuscript. To really improve this section, the authors could look for phosphorylation of lamin B1, which, if found to be p38 dependent, would somewhat clarify the mechanism.

11) Lastly, I am unclear why the authors switched from examining primarily fibroblasts to examining exclusively lymphoblasts in this figure (and then switched back to fibroblasts in Figure 6). For the sake of consistency, I suggest including some fibroblast data in this figure.

Figure 6:

12) The p38 silencing is very inefficient, particularly in GM02052. This leads to a decrease in lamin B1 that seems almost negligible via western, despite the quantitation results. I suggest repeating this experiment in this cell strain (at least) to generate better quality data. Alternatively, given how difficult it can be to transfect fibroblasts, the authors could simply use SB203580 to inhibit p38, as they do in Figure 5. SB203580 is potent and highly specific, and its effectiveness can be easily verified via phosphorylation of a downstream target of p38 such as ATF-2 or Hsp27.

Figure S1:

13) "Quantitative RT-PCR analysis did not reveal any increase of lamin B1 mRNA in A-T cells". In fact, it seems to reveal a decrease that looks statistically significant. To avoid the perception that the authors are misleading the reader, I suggest clearly stating that the mRNA levels are decreased, and offer a potential explanation as to what the cell gains by decreasing mRNA level only to increase protein level.

Figure S2:

14) It is unclear why the authors switched the order of lanes between the left and right side of (A). I suggest reorganizing the lanes on the right side to match the order on the left side. Presumably this can be done via cutting and pasting, leaving space between the lanes to demonstrate the manipulation - a new blot is not necessary.

15) While the increased phosphorylation of p38 in A-T cells is evident by the western blot, this is in seeming contradiction with at least two other reports that saw no increase in p38 phosphorylation in unchallenged A-T cells (Naka et al., 2004 from the author's bibliography and Davis T and Kipling D, 2009. *Biogerontology* 10: 253-266). The authors may wish to discuss this discrepancy.

Discussion and additional suggestions:

16) The authors' data suggest that lamin B1 increase should be associated with p38 activation in multiple settings, particularly in multiple types of senescence. To expand this finding into more general senescent phenotypes, I suggest that the authors examine lamin B1 levels in other types of senescence that are known to induce oxidative stress and activate p38, e.g. replicative senescence, direct DNA damage induced senescence (via irradiation - the authors seem to have access to an IR-generator) or oncogene-induced senescence such as Ras overexpression. A Ras-induced senescence experiment is particularly important, both because of widespread interest in oncogene-induced senescence and because Ras is mediated primarily by oxidative stress (like A-T senescence) and yet existing Ras data may not support the authors' results: Mason, et al (Mason DX, et al., 2004. *Oncogene* 23: 9238-9246) performed an extensive microarray analysis of Ras-induced gene expression changes in primary fibroblasts and saw an average six-fold decrease in LMNB1 mRNA levels (see supplementary data table - protein levels were not examined). Ras expression, of course, has been extensively demonstrated to induce reactive oxygen species and p38MAPK activation, which should stabilize lamin B1, according to the authors.

17) The authors claim a role for lamin B1 specifically, rather than a role for lamins generally. Given that many lamin B1 antibodies cross-react with lamin B2, and given the known role of lamin A/C dysregulation in senescence and progeroid syndromes, it would be useful to:

- Verify the data with a second lamin B1 antibody in at least one case (this is also important because of the unexpected correlation between decreased mRNA yet increased protein level of lamin B1).
- Include at least one control showing that the authors' interventions (such as H<sub>2</sub>O<sub>2</sub> treatment, NAC treatment, p38 activation, or p38 inhibition) do not alter the levels of lamin A/C.
- Test whether the overexpression of other lamins has the same effect as lamin B1 overexpression

Referee #3 (Remarks to the Author):

Title: ATM-alternative senescence pathway induced by lamin B1 accumulation, in response to oxidative stress.

Authors: Barascu et al.

It is widely accepted that ATM deficiency generates increased oxidative stress. However, it is not clear whether ATM is a direct sensor of the redox state or whether accumulation of unrepaired DNA damage that alters the cellular metabolism is the main cause for the elevated oxidative stress. As stated by the authors, malfunctioning DDR, oxidative stress and nuclear shape alterations are highly interconnected and play a role in senescence. Based on this notion the authors analyzed alterations in ATM deficiency-induced nuclear shape alterations for potential causes of senescence. They found accumulation of lamin B1 and alterations in nuclear shape in ATM-deficient cells. Over-expression of lamin B1 was sufficient to induce alterations in nuclear shape in WT cells. In parallel they found that oxidative stress was sufficient to cause lamin B1 over-expression and alterations in nuclear shape. The authors also showed that lamin B1 levels were controlled by p-38 MAPK. This is a well written paper that presents novel and interesting findings. However, this work despite being interesting is too descriptive at this point and lacks mechanistic understanding of the pathway. In addition, there are some questions and concerns that must be fully addressed before considering it for publication in EMBO J.

Main points:

1. In the current manuscript the authors focused on two cell lines: fibroblasts and lymphoblasts. These are mitotic cells that can undergo senescence. To generalize this study I would suggest that the authors analyze the effect of ATM deficiency on lamin B1 in post-mitotic neuronal cells. If indeed lamin B1 levels are upregulated in ATM-deficient neuronal cells then it might have other functions in addition to those involved in senescence. This will particularly be interesting in Purkinje neurons in which the amount of heterochromatin is rather low in ATM-deficient cells and there are no obvious alterations in nuclear shapes.
2. Is accumulation of unrepaired DNA sufficient to induce lamin B1 expression?
3. It would be helpful if the authors could provide additional data regarding the mechanism by which lamin B1 over-expression inhibits cell division?
4. Figure S1, in which ATM-dependent lamin B1 over-expression is not dependent on transcription, should be part of the article and not part of the supplementary data. Based on these data the authors claim that ATM deficiency increases the stability of lamin B1. It is necessary that the authors show the mechanism by which ATM deficiency stabilizes lamin B1. Is it reduced proteolysis? Are the ubiquitin system or lysosomes involved? Further molecular details are needed to delineate this phenomenon.
5. The authors used anisomycin as a p38-MAPK activator. However, anisomycin is also a very potent protein synthesis inhibitor. In this respect, this experiment is a replication of the cycloheximide experiment.
6. Will increased oxidative stress in the absence of lamin B1 cause senescence?
7. Figure 2: I would suggest that the authors present higher magnification photographs of the altered shape nuclei and better describe the criteria used to determine abnormal nuclear shape. This also applies to Figure 4.
8. The authors should show the effects of NAC on lamin B1 expression in WT cells.

Minor points:

1. For the general reader please provide the information that BSO causes oxidative stress through the inhibition of GSH synthesis.
2. Page 15, third line: DRR should be DDR.
3. Page 17, line 13: Anti-p(S1982)-ATM should be anti-p(S1981)-ATM.

Dear Editor

Thank you for giving us the opportunity to send you a revised version of the manuscript newly entitled: "**ATM-alternative senescence pathway induced by lamin B1 accumulation through p38 MAPK activation in response to oxidative stress**"

We thank the reviewers. Addressing their comments substantially improved the manuscript, and we expect that it is now suitable for publication in *EMBO Journal*.

The following is a list of the experiments that were added to the revised version of the manuscript:

1. We used the Cell Profiler software to monitor the circularity of nuclei and analyze nuclear morphology for all data, under different conditions (i.e, A-T vs. WT, Oncogene- or stress-induced senescence, overexpression of lamin B1 and following different cell treatments) (Figures 2, 4, 5, 8, and Figure S8).
2. Using siRNAs against lamin B1, we showed that decreasing lamin B1 levels rescues both nuclear morphology and senescence in A-T cells, indicating the actual involvement of lamin B1 in A-T cell senescence. (Figure 4).
3. We showed that inhibition of p38 MAPK activity prevented senescence and high lamin B1 levels induced by H<sub>2</sub>O<sub>2</sub> (Figure S7).
4. We establish the IR-induced senescence model in the WI38 cell line and measured the levels of lamin B1, nuclear shape alterations (NSAs) and senescence frequencies in this model (Figure S8 A). These experiments showed an increase of lamin B1 and NSAs in this senescence condition.
5. We used an OIS senescence model (ER-RasV12 expressing IMR 90 cells kindly provided by Dr. Narita) and measured the levels of lamin B1, nuclear shape alterations and senescence frequencies in this model (Figure S8 B).

These experiments showed an increase of lamin B1 and NSAs during stress-induced senescence and OIS, showing that lamin B1 accumulation is not restricted to A-T and suggesting lamin B1 accumulation as a general marker or mediator of senescence.

6. We showed an *in vitro* interaction between p38 MAPK and lamin B1 using co-immunoprecipitation experiments and an *in situ* interaction between p38 MAPK and lamin B1 using the proximity ligation assay (DuoLink) (Figure 7 C).
7. We showed the *in vitro* phosphorylation of lamin B1 by p38 MAPK (Figure 7D).
8. We measured basal levels and H<sub>2</sub>O<sub>2</sub>-induced levels of ROS after lamin B1 overexpression. We compared survival after H<sub>2</sub>O<sub>2</sub> treatment between cells transfected with the control vector and lamin B1 vector (Figure S9). Basal ROS and induced-ROS

- were decreased and cell survival was improved upon lamin B1 overexpression, showing a protective role of lamin B1 against oxidative stress.
9. We showed that differences in lamin B1 stabilization between A-T and WT cells are not due to apoptosis using cycloheximidine combined with Z-VAD, as similar data were obtained in the presence of this apoptosis inhibitor. The efficiency of the Z-VAD treatment was verified by measuring PARP1 cleavage (Figure S5 B).
  10. We measured the levels of lamin B1 after MG132 or leupeptin treatment (Figure S5 C), showing a participation of proteasome for the regulation of lamin B1 turn-over.
  11. We verified the efficiency of cycloheximide treatment using caspase as a control (Figure S5A).
  12. We showed that DDR is not activated upon lamin B1 overexpression by measuring the levels of phosphor-Chk1, phosphor-Chk2, and  $\gamma$ -H2AX. The number of  $\gamma$ -H2AX foci was also quantified (Figure S3).
  13. We showed the loss of proliferation upon lamin B1 overexpression by BrdU incorporation and WB detection of cyclin A (Figure 3 D).
  14. We confirmed the high levels of lamin B1 protein in A-T cells using an additional antibody raised against lamin B1 (Figure S1).
  15. We measured the immunofluorescence intensity of lamin B1 in A-T nuclei, confirming the overexpression of lamin B1 (Figure S2).
  16. We measured the levels of lamin A/C and lamin B2 in A-T cells (Figure S1), showing that only lamin B1 is increased in A-T cells. We showed also that levels of lamin A/C were unchanged after NAC treatment in A-T (Figure S4B).
  17. Anisomycin treatment was performed in primary fibroblasts (Figure S6D), showing that lamin B1 is also increased upon MAPK activation in these cells, as it was observed in lymphoblasts.
  18. We verified the efficiency of anisomycin treatment by measuring the activation of P-p38 MAPK (Figure 7A).
  19. We verified the efficiency of the treatment with the p38 inhibitor SB203580 by measuring the level of phosphorylated HSP27, a target of p38 MAPK (Figure 7B).



## **ANSWERS to EDITOR's comments.**

Furthermore, the key points for a successful revision in this case would be the following:

**1)** - significantly improving the assessment and scoring of nuclear shape changes - this is a key foundation of the manuscript and I feel that especially the experts from the nuclear lamina side will need to be convinced here [see referee 1's first 'specific comment', referee 2's point 4 (including useful scoring suggestions) and referee 3's point 7]

As suggested by referee 2, we used the Cell Profiler software to measure the circularity of the nuclei. We described the module used in the Materials and Methods section and page 6-7 of the manuscript. We used the software to analyze nuclear shape alterations in the different situations described in the paper as follows: A-T vs. WT, overexpression of lamin B1, in OIS or stress-induced senescence and following different cell treatments (pro-oxidant, anti-oxidant, inhibition of MAPK-P38, siRNA against lamin B1) (Figures 2, 4, 5, 8, and S8).

**2)** - adding at least some mechanistic insight into the mechanism of lamin B1 stabilization by p38, such as p38-mediated phosphorylation or proteasome/lysosome inhibition experiments [referee 2 point 10, referee 3 point 4]

**A-** First, as suggested by referees 2 and 3, we analyzed the impact of proteasome or lysosome inhibition (with MG132 or leupeptin treatment) on lamin B1 levels. Treatment with the proteasome inhibitor MG132 (but not leupeptin) increased the level of lamin B1 in wild-type cells, suggesting that at least part of the turnover regulation of lamin B1 involved the proteosomal pathway (Figure S5 C).

**B-** Second, we described an interaction between p38 MAPK and lamin B1 using two different approaches: co-immunoprecipitation and PLA (DuoLink) experiments. This latter approach allowed us to detect endogenous interactions *in situ* and in close proximity ( $\leq 40$  nm) (Figure 7 C).

**C-** Third, we showed that *in vitro* p38 MAPK is able to phosphorylate lamin B1 (Figure 7D).

**3)** - adding missing experiments to bolster the causality (rather than simply correlation) in some instances [referee 3 point 6: do ROS still cause senescence in absence of lamin B1; referee 2 point 7: does lamin B1 loss in A-T cells cause the same rescue effects as p38 inhibition or ROS scavenging]

Here we show the following:

**A-** The extinction of p38 MAPK in A-T cells rescues lamin B1 levels, nuclear shape alterations and senescence (Figure 8);

**B-** The decrease in lamin B1 levels in A-T cells (to levels comparable with lamin B1 levels in WT cells) prevents nuclear shape alterations and senescence, indicating that lamin B1 accumulation in A-T cells is involved in senescence (Figure 4).

**4)** - related to this is the issue of generalization: confirm that p38 inhibition or lamin B1 loss rescue senescence induced by ROS treatment or ATM inhibition also in wild-type cells [referee 1's first major point] and test if lamin B1 overexpression and nuclear alterations would also occur in at least one other form of ROS- and p38-involving senescence, such as Ras-induced senescence [referee 2 point 16]

**A-** We also show in this revised version that the inhibition of p38 MAPK prevents both the accumulation of lamin B1 and senescence induced by H<sub>2</sub>O<sub>2</sub> (Figure S7).

**B-** Thus, it is tempting to propose that lamin B1 is a general mediator and marker of senescence induced by OS.

To strength this suggestion, we analyzed lamin B1 levels and nuclear shape alterations in 2 canonical senescence processes:

- OIS induced by expression of the Ras oncogene (cellular model developed and kindly provided by Dr Narita);
- a model of stress-induced senescence that was established in our laboratory (using conditions described by Campisi's laboratory; see Freund et al, 2011, EMBO J, 20:1536-48) by exposing WI 38 cells to 10 Gy radiation.

Nuclear morphology alterations and lamin B1 levels increase during these 2 canonical situations of senescence, in which P38 MAPK is also induced. These data show that lamin B1 accumulation is generally associated with senescence in response to ROS induction (Figure S8). These important data are in the supplementary section due to space limitations.

In the revised version, we added additional data and one point of discussion that were not directly asked but that we believe are very relevant to the general message of our manuscript. We described a protective role of the increase in lamin B1 against oxidative stress in wild-type cells. In fact, we showed that lamin B1 overexpression protects cells against endogenous and induced ROS and improves cell survival (Figure S9). This underlines that lamin B1 levels should be tightly regulated because prolonged higher levels or accumulation (as in A-T) could induce senescence and may be responsible for neurological defects (especially demyelination).

**5)** On the other hand, points that may be beyond the scope of the current analysis and where addressing them in writing may be sufficient (nevertheless, this will require diligent discussions of these issues), are the following:

- ref 2 point 6 (if difficult to do in your transfection settings)
- ref 2 point 17 (but any relevant control data you may have to answer this would be helpful)
- ref 3 point 1

**A-** ref 2 point 6 (if difficult to do in your transfection settings)

« Beta-gal staining and SAHF formation are both used as markers of senescence, but both have been shown to occur in the absence of growth arrest. It would be fairly quick to perform a 1-2 week growth curve or colony formation assay on lamin B1 overexpressing cells to determine whether they were actually growth arrested, and thus senescent. »

We performed BrdU incorporation analysis and analysis of cyclin A levels (Figure 3D), reflecting cells transitioning into S phase. These experiments gave more precisions than a curve growth and it was done in time course in which lamin B1 overexpression induces senescence. The cells overexpressing lamin B1 showed less BrdU incorporation and a decrease in cyclin A levels, indicating a loss of proliferation.

**B-** ref 2 point 17 (but any relevant control data you may have to answer this would be helpful)  
« The authors claim a role for lamin B1 specifically, rather than a role for lamins generally. Given that many lamin B1 antibodies cross-react with lamin B2, and given the known role of lamin A/C dysregulation in senescence and progeroid syndromes, it would be useful to:  
-Verify the data with a second lamin B1 antibody in at least one case (this is also important because of the unexpected correlation between decreased mRNA yet increased protein level of lamin B1).  
-Include at least one control showing that the authors' interventions (such as H<sub>2</sub>O<sub>2</sub> treatment, NAC treatment, p38 activation, or p38 inhibition) do not alter the levels of lamin A/C.

We included data showing that 2 different antibodies raised against lamin B1 produced the same results and showed that lamin B2 and A/C did not increase in A-T primary fibroblasts (Figure S1). Moreover, lamin A/C levels in A-T cells did change upon NAC treatment (Figure S4).

**B-** ref 3 point 1

« In the current manuscript the authors focused on two cell lines: fibroblasts and lymphoblasts. These are mitotic cells that can undergo senescence. To generalize this study I would suggest that the authors analyze the effect of ATM deficiency on lamin B1 in post-mitotic neuronal cells. If indeed lamin B1 levels are upregulated in ATM-deficient neuronal cells then it might have other functions in addition to those involved in senescence. This will particularly interesting in Purkinje neurons in which the amount of heterochromatin is rather low in ATM-deficient cells and there are no obvious alterations in nuclear shapes. »

We agree that these studies would be interesting. Because, lamin B1 plays a major role in such an important cell structure that is the nucleus, it is conceivable that overexpression might affect other cellular functions. Since, the level of heterochromatin is low in Purkinje neurons, might suggest that nuclear malformation may be correlated to the level of heterochromatin.

However, this point deserve deep investigations, it is time consuming and will require space in manuscript that is beyond the size limitations when considering the entire data set from the first version plus the additional experiments requested by the reviewers. In fact, this point deserves one specific study and one specific manuscript. We did attempt to obtain tissues to begin addressing this issue, but we did not obtain sufficient quantities of good-quality tissues for consistent analysis.

## **ANSWERS to REVIEWERS' comments.**

### **Referee #1 (Remarks to the Author):**

**1)** « One critical experiment is to test whether e.g. H<sub>2</sub>O<sub>2</sub>- or ATM inhibition-mediated senescence in wt cells can be rescued by p38 downregulation/inhibition or lamin B1 downregulation. »

As discussed above in the ANSWERS to the EDITOR's comments, we added the following new data:

**A-** Inhibition of P38 MAPK prevents the accumulation of lamin B1 levels and senescence induced by H<sub>2</sub>O<sub>2</sub>, showing that lamin B1 is associated with senescence induced by oxidative stress (Figure S7).

**B-** Decreasing lamin B1 levels in A-T cells by RNA interference (to levels comparable with levels in WT cells) prevents nuclear shape alteration and senescence, showing that lamin B1 accumulation in A-T is involved in senescence (Figure 4).

**2)** « Furthermore, to show that lamin B1 overexpression is indeed downstream of oxidative stress, DNA damage and p38 kinase it is important to show that lamin B1 overexpression does not affect these parameters. »

We included and discussed the following data to address this point:

**A-** DDR activation was not observed after lamin B1 overexpression. No changes in p-Chk1, p-Chk2 and  $\gamma$ -H2AX levels (evaluated by western blot) or the number of  $\gamma$ -H2AX foci (monitored by immunofluorescence microscopy) were observed 48 H post-transfection, which corresponds to the time that senescence was detected (Figure S3). This suggests that lamin B1 overexpression does not induce senescence through DNA damage, and therefore, lamin B1 acts downstream of DNA stress to induce senescence.

**B-** Overexpression of lamin B1 did not increase oxidative stress or p38 kinase. In contrast, overexpression of lamin B1 results in a decrease in ROS and a concomitant decrease in p38 MAPK. A decrease in H<sub>2</sub>O<sub>2</sub>-induced ROS accompanied by improved cell survival was also observed (Figure S9). These data show that lamin B1 overexpression is part of the cell's response to OS and are consistent with data from the laboratory of D. Vaux, which proposed the transcriptional regulation of detoxification genes by lamin B1 via the retention of Oct1.

Our data show an important role for lamin B1 in the control of oxidative stress.

Altogether, these data show that lamin B1 acts downstream of oxidative stress, DNA damage and p38 MAPK to induce senescence. These data support our model presented in Figure 9.

Specific comments:

**3)** « Immunofluorescence microscopy is of poor quality and nuclear shape changes shown in Fig. 2 and 4 are not convincing. »

We added representative images of nuclear changes in Figure 2. As discussed in the ANSWERS to EDITOR's comments, we used the Cell Profiler software to objectively measure the circularity of the nuclei. We described the module used in the Materials and Methods section and page 7 of the manuscript. We used the software to analyze nuclear shape alterations in the different conditions described in the paper (Figures 2, 4, 5 (Fig 4 of previous version), 8 and S8). Of note, the data obtained using the software are very similar to what we had previously observed "by eye". For instance, we detected 48 and 55% deformed nuclei in A-T cells (GM 05823 and GM 02052, respectively) by eye compared with 57.5 and 48.1% using the software (circularity  $\leq 0.65$ ) as well as 11 and 14% in wild-type cells (GM 03348 and GM 05757, respectively) by eye compared with 16.8 and 16.6% using the software (circularity  $\leq 0.65$ ).

**4)** Fig. 1: Is this effect specific for lamin B1 or are other lamins affected as well. Blots for lamin B2 and lamins A/C should be provided.

**A-** Lamin A, C, and B2 were analyzed in A-T cells (Figure S1). Lamin B1 was exclusively overexpressed in A-T cells; lamin A, C and B2 levels did not increase.

**B-** NAC treatment did not change the levels of lamin A/C but did change lamin B1 levels (Figure S4B).

**5)** Fig. 5: A control should be added showing degradation of unrelated proteins in AT cells. I am surprised that actin is not turned over at all in 9h.

We measured the caspase level by western blot as a control. The caspase levels decreased upon cycloheximide treatment (relative to lamin B1 levels), showing the efficiency of this treatment in A-T cells (Figure S5A).

**4)** Fig. 5B: Activation or inhibition of p38 / MAPK signaling should be demonstrated.

**A-** We verified anisomycin treatment efficiency by measuring the activation of P-p38 MAPK (Figure 7A).

**B-** We verified SB203580 (p38 inhibitor) treatment efficiency by measuring the levels of phosphorylated HSP27, a target of p38 MAPK (Figure 7B).

## Referee #2 (Remarks to the Author):

Comments for transmission to the authors

In this manuscript, Barascu et al present data to show that lamin B1 accumulates in cells lacking functional ATM. This lamin B1 accumulation is sufficient to induce cellular senescence, as evidenced by beta-gal staining, heterochromatic foci, and misshapen nuclei. The authors demonstrate that this lamin B1 increase is dependent on oxidative stress, which activates p38, which in turn stabilizes the protein level of lamin B1. The authors conclude that their data connect the two classes of progeroid syndromes (laminopathies and DDR defect syndromes). Research into cell cycle regulation and gene expression regulation by the nuclear periphery is an area of increasing interest - the authors' results are mostly novel, and if they prove to be generally applicable, they would attract a wide readership,

but the paper would benefit if the authors expanded their findings into multiple forms of senescence ....

To expand our findings into multiple situations of senescence, as suggested by this referee, we analyzed the lamin B1 levels and nuclear shape alterations during

- A-** OIS induced by expression of the Ras oncogene (cellular model developed by Dr. Narita).
- B-** Stress-induced senescence using a model we established in the laboratory (conditions described by Campisi 's laboratory, see Freund et al, 2011, EMBO J, 20:1536-48) by exposing WI 38 cells to 10 Gy of radiation.

Nuclear morphology alterations and lamin B1 levels increased during these 2 canonical situations of senescence in which p38 MAPK is also induced (Figure S8). These data show that lamin B1 accumulation is generally associated with senescence in response to ROS.

Additionally, there are several control experiments that would significantly increase the reliability of the conclusions, as I enumerate below.

Introduction: In general, this is a good introduction to senescence and lamin biology and adequately covers the appropriate literature. A few criticisms:

**1)** There are more grammatical and syntactical errors in the Introduction than in following sections. I suggest that the authors have a native English speaker perform a line-by-line edit of at least this section.

English editing of the revised version was performed by American Journal Experts.

**2)** "...senescence is responsible for organism aging...": While this is a hypothesis that is prevalent in the field of senescence, it is far from being proven or even directly supported. At best, the evidence is correlative. I suggest toning down the language and stating that this is a hypothesis.

In the revised version, we wrote "beside its putative role in organism ageing" instead of "...senescence is responsible for organism aging..."

**3)** While there is not a lot of research into lamin B1 out there, there are several studies examining lamin B1 loss, and these data should be discussed, as it seems that lamin B1 loss phenocopies many of the effects the authors see with lamin B1 accumulation, which is

surprising. For example, here are a few papers that suggest that loss of lamin B1 induces premature senescence and misshapen nuclei:

- Lammerding J, et al., (2006) J Biol Chem 281: 25768-25780
- Vergnes L, et al., (2004) Proc Natl Acad Sci U S A 101: 10428-10433

These papers do not conflict with the authors' data directly; however it is surprising that both the accumulation and the depletion of a protein induce similar phenotypes.

We agree with the referee and discuss this point on pages 9 and 17 of the manuscript. Both low and high levels of lamin B1 affect nuclear architecture and lead to senescence (references cited above and unpublished observations from our laboratory). These data suggest that a precise balance of lamin B1 is required for nuclear morphology integrity and to avoid senescence.

Figure 1: good: the authors examine multiple cell strains and tissue types. I have no critiques

Figure 2:

**4)** How was nuclear morphology assessed? If this was done by eye, it strikes me as highly subjective. An example of objective quantitation would be to use an image analysis software like CellProfiler (cellprofiler.org) to analyze the negative curvature of the nucleus, determine a curvature score, and set a threshold to define normal vs abnormal (or even better - compare the two histograms directly rather than making a binary distinction). This analysis could be performed on existing images - no new experiments necessary. At the same time, it would be interesting to quantitate lamin B1 level by IF and determine if there is a correlation between lamin B1 level and nuclear curvature/abnormality, as one would predict from the authors' data.

As already discussed above in the ANSWERS to EDITOR's comments and in a response to the comments to referee 1, we used the Cell Profiler software to measure the circularity of the nuclei. We would like to thank this reviewer for his useful suggestion. We described the module used in the Materials and Methods section and page 7 of the manuscript. We used the software to analyze nuclear shape alterations in the different conditions described in the paper (Figures 2, 4, 5, 8, and S8). We should note that the data obtained using the software were very similar to the data previously obtained "by eye" (for examples, see the answer to the referee 1's point **3**) comments).

**5)** « A western blot showing the level of lamin B1 overexpression would be useful here as a supplement, as would a western blot showing the level of lamin A/C in wild-type versus A-T cells, because the authors are claiming a role for lamin B1 specifically rather than a role for lamins in general.

**A-** We performed western blot analysis of lamin A/C and B2 in A-T cell extracts (Figure S1).

Lamin B1 overexpression was exclusively overexpressed in A-T cells; lamin A, C and B2 levels did not increase.

**B-** Moreover, in Figure S4, NAC treatment did not change the levels of lamin A/C.

Additionally, why do the authors use lamin A/C in (a) but lamin B1 in (b) - this inconsistency is

needlessly confusing.

In Figure 2A, we changed the images and now show nuclear deformation using the lamin B1 antibody.

Figure 3:

**6)** Beta-gal staining and SAHF formation are both used as markers of senescence, but both have been shown to occur in the absence of growth arrest. It would be fairly quick to perform a 1-2 week growth curve or colony formation assay on lamin B1 overexpressing cells to determine whether they were actually growth arrested, and thus senescent.

Colony formation assays are difficult to set up in our experimental conditions. To address this point more precisely, we performed BrdU incorporation analysis and WB analysis of cyclin A to detect cells transitioning into S phase. The cells overexpressing lamin B1 showed less BrdU incorporation and a decrease in cyclin A levels, indicating a loss of proliferation. Thus, senescence upon lamin B1 overexpression was evaluated by 3 different senescence features: SA- $\beta$ -gal staining, SAHF formation and proliferation loss.

**7)** « As lamin B1 overexpression in normal cells induces beta-gal and aberrant nuclear shape, it would provide symmetry to test whether lamin B1 depletion in A-T cells delays beta-gal staining and reduces aberrant nuclear shape. Additionally, this would support the subsequent conclusions that NAC and SB203580, which reduce beta-gal and aberrant nuclear morphology (this is well known), accomplish this delay by decreasing lamin B1. This would also address the seeming discrepancy with previously published literature, as I discussed in point #3. »

In the revised version, we show that decreasing lamin B1 levels by RNA interference in A-T prevents both nuclear shape alterations and senescence, indicating that lamin B1 accumulation in A-T cells is involved in senescence (Figure 4). Of note, the siRNA efficiency was partial, and the lamin B1 levels were comparable to WT levels. This point is essential because, as underlined by this referee (see above point 3 of this comment), a lower level of lamin B1 could lead to nuclear shape alteration and potentially to senescence.

« Figure 4: mostly good. I appreciate that the authors looked at both cell types and multiple inducers of ROS.

**8)** A minor point is that the lamin B1 levels in NAC-treated A-T cells look higher than the lamin B1 levels of untreated A-T cells in (c) - in fact, they look as high as the lamin B1 levels of H<sub>2</sub>O<sub>2</sub> treated wild-type cells. While IF is not particularly quantitative, the trend certainly shouldn't be opposite what is seen with the western data in (a).

Here, it is difficult to compare the fluorescence intensity between the different image fields corresponding to different treatments. The images are used here to determine the circularity of the nuclei. The set up of the fluorescence intensity was chosen to have a sharp contour of the nuclei for the circularity analysis. If the fluorescence is too low, the software cannot easily determine the contours. We should note that western-blot analysis is still the best way to



appreciate the lamin B1 levels in this case. Anyway, we changed images for NAC treatment of A-T to show more representative images.

« Figure 5:

**9)** Using cycloheximide to prevent protein translation and determine protein stability is not uncommon; however, the results must be interpreted with caution. Cycloheximide is a potent inducer of apoptosis (e.g. Blom WM, et al., 1999. *Biochem Pharmacol* 58: 1891-1898). The concentration and time is cell-type dependent, but 3-9 hours at 50 ug/mL is well within the range of many published studies. Apoptosis, of course, is characterized by lamin B1 cleavage and degradation (e.g. Kivinen K et al., 2005. *Exp Cell Res* 311: 62-73). Because A-T cells are resistant to apoptosis (e.g. 2nd paragraph of Cosentino et al., 2011 in the author's bibliography), it is conceivable that the difference in protein stability seen between A-T cells and wild-type cells is simply because A-T cells are more resistant to cycloheximide-induced apoptosis and thus lamin B1 degradation. To test this possibility, the authors could repeat the experiment in the presence of Z-VAD, which is a caspase-3 inhibitor. If Lamin B1 is still decreased in the presence of cycloheximide, it suggests a true stability shift. »

In the revised version, we performed the suggested experiments. We showed that differences in lamin B1 stabilization between A-T and WT cells is not due to differences in apoptosis, as similar data were obtained in the presence of the apoptosis inhibitor Z-VAD. In this experiment, the Z-VAD treatment efficiency was verified by measuring PARP1 cleavage (Figure S5 B).

**10)** "I don't understand why the authors state that p38MAPK seemed like a good candidate for lamin B1 regulation (page 9, bottom), given the change in protein stability. To my knowledge there are zero published studies connecting lamin B1 regulation to p38MAPK, and p38MAPK is not generally reported to affect protein stability - it primarily regulates mRNA stability and transcription. Some explanation of how the authors think p38MAPK can affect protein stability at all, let alone lamin B1 stability, would be useful. To really improve this section, the authors could look for phosphorylation of lamin B1, which, if found to be p38 dependent, would somewhat clarify the mechanism. »

We believed that p38 MAPK was a good candidate because 1) it is a kinase activated by oxidative stress; 2) it is activated during senescence induced by different stresses; and 3) perhaps more importantly, it has also been proposed as part of an alternative ATM pathway of senescence.

To add mechanistic insight into the mechanism of lamin B1 stabilization by p38, we described :

**A\_** an interaction between p38 MAPK and lamin B1 using co-immunoprecipitation and PLA (DuoLink) experiments. The latter approach allows the detection of endogenous interactions *in situ* and in close proximity ( $\leq 40$  nM) (Figure 7 C).

**B-** Importantly, we showed that *in vitro* MAPK-P38 is able to phosphorylate lamin B1 (Figure 7D).

**11)** Lastly, I am unclear why the authors switched from examining primarily fibroblasts to examining exclusively lymphoblasts in this figure (and then switched back to fibroblasts in Figure 6). For the sake of consistency, I suggest including some fibroblast data in this figure.

In the revised version, we performed western blot analysis and showed that lamin B1 levels increase by anisomycin treatment in wild-type primary fibroblasts as it was observed in lymphoblasts (Figure S6D).

Figure 6:

**12)** The p38 silencing is very inefficient, particularly in GM02052. This leads to a decrease in lamin B1 that seems almost negligible via western, despite the quantitation results. I suggest repeating this experiment in this cell strain (at least) to generate better quality data. Alternatively, given how difficult it can be to transfect fibroblasts, the authors could simply use SB203580 to inhibit p38, as they do in Figure 5. SB203580 is potent and highly specific, and its effectiveness can be easily verified via phosphorylation of a downstream target of p38 such as ATF-2 or Hsp27.

- A-** We included data showing more efficient p38 knockdown by siRNA in the GM02052 cells (A-T cells) (changes in Figure 8A).
- B-** In addition, we included data showing that SB203580 treatment rescued the nuclear shape and senescence induced by H<sub>2</sub>O<sub>2</sub> treatment (Figure S7).
- C-** As suggested, we verified the effectiveness of SB203580 by measuring phosphor-Hsp27 levels (Figure 7B).

Figure S1:

**13)** "Quantitative RT-PCR analysis did not reveal any increase of lamin B1 mRNA in A-T cells". In fact, it seems to reveal a decrease that looks statistically significant. To avoid the perception that the authors are misleading the reader, I suggest clearly stating that the mRNA levels are decreased, and offer a potential explanation as to what the cell gains by decreasing mRNA level only to increase protein level.

The difference in mRNA levels between A-T and WT cells are mainly significant, as measured by t-tests. These data suggest that a negative feedback loop controls excessive levels of lamin B1, which was previously suggested in oligodendrocytes in which miRNA expression control the levels of lamin B1 (Lin et al, 2009, Dis Model Mech. 2: 178–188). The histograms were transferred to Figure 6 A of the revised manuscript instead, and this point is discussed in the revised manuscript (page 11).

Figure S2:

**14)** It is unclear why the authors switched the order of lanes between the left and right side of (A). I suggest reorganizing the lanes on the right side to match the order on the left side. Presumably this can be done via cutting and pasting, leaving space between the lanes to demonstrate the manipulation - a new blot is not necessary.

We reorganized the lanes, as the referee proposed, and carefully separated the edited portions of the blot to clearly show the manipulation. These data are now in Figure S6 B of the

revised version.

**15)** While the increased phosphorylation of p38 in A-T cells is evident by the western blot, this is in seeming contradiction with at least two other reports that saw no increase in p38 phosphorylation in unchallenged A-T cells (Naka et al., 2004 from the author's bibliography and Davis T and Kipling D, 2009. *Biogerontology* 10: 253-266). The authors may wish to discuss this discrepancy.

It is well established that unchallenged A-T cells exhibit higher OS, on which therapeutic strategies of A-T patients using anti-oxidants are based. It was shown that anti-oxidant feeding decreases frequency of lymphoma, increases the life span and improves neurological behavior in A-T mice. As p38 MAPK is activated by OS, it was not unexpected to find activation of this protein in A-T cells.

The authors of the first study listed (Naka et al., 2004) established hTERT-expressing A-T fibroblasts. In 2 cell lines, they could not detect the activation of p38 using an antibody raised against phosphor-p38. However, the authors still proposed that the p38 MAPK pathway induces senescence in A-T cells. In the second study (Davis et al., 2009), p38 MAPK activation was not observed in 2 human primary fibroblast lines. However, in our study, use of the p38 inhibitor SB203580 increased the life span of both fibroblast lines. Thus, both studies suggested that p38 MAPK plays a role in senescence in A-T-deficient cells, although downstream effectors were not identified.

In both studies, only western blot analysis was performed to analyze p38 activation. In our study, we showed an increase in p38 MAPK activation in unchallenged A-T cells using 2 different approaches: (1) measuring the phosphorylated form of p38 (corresponding to the active form) by western blot in 2 different A-T lymphoblast cell lines and 2 different A-T primary fibroblast cell lines (figure S6 A); and (2) measuring on ATF2, a specific target of p38, the actual kinase activity of immunoprecipitated p38 from 2 A-T lymphoblast and 2 wild-type lymphoblast lines (figure S6 B).

Finally, other studies show the activation of p38 in different ATM-deficient cells (in hematopoietic stem cells: Ito et al., 2006, *Nat Med*,12:446-51; in neural stem cells: Kim et al, 2009. *Stem cells*, 27:1987-98; in marrow stromal cells: Hishiya et al., 2005, *Bone*, 37:497-503).

Discussion and additional suggestions:

**16)** The authors' data suggest that lamin B1 increase should be associated with p38 activation in multiple settings, particularly in multiple types of senescence. To expand this finding into more general senescent phenotypes, I suggest that the authors examine lamin B1 levels in other types of senescence that are known to induce oxidative stress and activate p38, e.g. replicative senescence, direct DNA damage induced senescence (via irradiation - the authors seem to have access to an IR-generator) or oncogene-induced senescence such as Ras overexpression. A Ras-induced senescence experiment is particularly important, both because of widespread interest in oncogene-induced senescence and because Ras is mediated primarily

by oxidative stress (like A-T senescence) and yet existing Ras data may not support the authors' results: Mason, et al (Mason DX, et al., 2004. *Oncogene* 23: 9238-9246) performed an extensive microarray analysis of Ras-induced gene expression changes in primary fibroblasts and saw an average six-fold decrease in LMNB1 mRNA levels (see supplementary data table - protein levels were not examined). Ras expression, of course, has been extensively demonstrated to induce reactive oxygen species and p38MAPK activation, which should stabilize lamin B1, according to the authors.

We analyzed lamin B1 levels and nuclear shape alterations:

- **A-** during senescence induced by expression of Ras oncogene (cellular model developed by Dr. Narita);
- **B-** in a model of stress-induced senescence that we established in the laboratory (with conditions described by Campisi' laboratory in Freund et al, 2011, *EMBO J*, 20:1536-48) by exposing WI 38 cells to 10 Gy of radiation.

NSA and lamin B1 levels increased during these 2 canonical situations of senescence, in which p38 MAPK is also induced (Figure S8). These data show that lamin B1 accumulation is more generally associated with senescence in response to ROS induction.

In the paper cited by the referee, Mason et al. observed a decrease in LMNB1 mRNA levels, but they did not analyze the protein levels. Here, we show a decrease in lamin B1 mRNA levels in A-T cells (Figure 6), while lamin B1 protein levels conversely increased. This apparent discrepancy between the mRNA and protein levels of lamin B1 in A-T cells and during oncogene-induced senescence could, in fact, reveal a negative feedback regulation loop. Of note, expression of miRNA affecting lamin B1 mRNA levels in oligodendrocytes was reported (Lin et al, 2009, *Dis Model Mech.* 2: 178–188), indicating a potential cellular response against an excess of lamin B1.

**17)** The authors claim a role for lamin B1 specifically, rather than a role for lamins generally. Given that many lamin B1 antibodies cross-react with lamin B2, and given the known role of lamin A/C dysregulation in senescence and progeroid syndromes, it would be useful to:

- Verify the data with a second lamin B1 antibody in at least one case (this is also important because of the unexpected correlation between decreased mRNA yet increased protein level of lamin B1).
- Include at least one control showing that the authors' interventions (such as H2O2 treatment, NAC treatment, p38 activation, or p38 inhibition) do not alter the levels of lamin A/C.

**A-** In the revised version, we included data showing that neither lamin B2 nor A/C levels increased in A-T primary fibroblasts (Figure S1).

**B-** Moreover, 2 different antibodies against lamin B1 were also used and yielded the same results (Figure S1).

**C-** Finally, NAC treatment did not affect lamin A/C levels in A-T fibroblasts (Figure S4),

suggesting that the observed effect is specific to lamin B1.

- Test whether the overexpression of other lamins has the same effect as lamin B1 overexpression

This suggestion itself could represent another project. Moreover, there are already studies showing that overexpression of lamin A also alters the nuclear envelope and could lead to progeroid phenotype (Candelario et al, 2008, *Aging Cell*, 7:355-67; Volkova et al, 2011, *J Electron Microsc.* 60:57-71), indicating that a precise equilibrium is required and that the level of lamin A should also be tightly controlled. It might be interesting to analyze the impact of lamin A/C on oxidative stress regulation. However, NAC treatment did not affect lamin A/C levels in A-T fibroblasts, suggesting that lamin A/C levels are not sensitive to oxidative stress.

### Referee #3 (Remarks to the Author):

Main points:

**1)** In the current manuscript the authors focused on two cell lines: fibroblasts and lymphoblasts. These are mitotic cells that can undergo senescence. To generalize this study I would suggest that the authors analyze the effect of ATM deficiency on lamin B1 in post-mitotic neuronal cells. If indeed lamin B1 levels are upregulated in ATM-deficient neuronal cells then it might have other functions in addition to those involved in senescence. This will particularly interesting in Purkinje neurons in which the amount of heterochromatin is rather low in ATM-deficient cells and there are no obvious alterations in nuclear shapes.

This is indeed an interesting suggestion. As lamin B1 plays a major role in such an important cell structure that is the nucleus, its overexpression might have other functions. Since, the level of heterochromatin is low in Purkinje neurons, might suggest that nuclear malformation may be correlated with heterochromatin levels.

However, this point deserve deep investigations, it is time consuming and will require space in manuscript that is beyond the size limitations when considering the entire data set from the first version plus the additional experiments requested by the reviewers. In fact, this point deserves one specific study and one specific manuscript. We did attempt to obtain tissues to begin addressing this issue, but we did not obtain sufficient quantities of good-quality tissues for consistent analysis.

**2)** Is accumulation of unrepaired DNA sufficient to induce lamin B1 expression?

ATM is a key factor for DNA damage signaling but also participates in controlling oxidative stress (Guo et al, 2010, Science, 330:517-21; Cosentino et al, 2011, 30:546-55). Therefore, we cannot exclude that in parallel to OS unrepaired DNA could also participate in the increase of lamin B1 levels in A-T cells. This is an important question that could represent exciting challenges and future prospects.

First, NAC treatment rescues nuclear morphology and senescence, which underlies the importance of OS in the end points measured here.

Second, initially we identified the overexpression of lamin B1 in A-T cells by a differential proteomic analysis. In parallel, we also performed a differential proteomic analysis of ligase 4-deficient cells (which are defective in DNA double-strand break (DSB) repair by Non Homologous End Joining (NHEJ) and are sensitive to ionizing radiation); we did not find any change in lamin B1 levels in ligase 4-deficient cells. We then confirmed this finding by western blot analysis.

Nevertheless, this point is very important, and we are currently investigating the status of lamin B1 in different DNA repair syndromes. While lamin B1 is overexpressed in some of them, it is not overexpressed in all DNA repair syndromes analyzed; thus, the situation appears

more complex.

These observations at least suggest that the presence of damage is not sufficient to induce lamin B1 in unchallenged DSB repair-defective cells.

**3)** It would be helpful if the authors could provide additional data regarding the mechanism by which lamin B1 over-expression inhibits cell division?

We performed BrdU incorporation analysis and Western Blot analysis of cyclin A. Cells overexpressing lamin B1 showed less BrdU incorporation and a decrease in cyclin A levels, indicating a loss of proliferation, by decreasing number of cells transitioning to S phase.

**4)** Figure S1, in which ATM-dependent lamin B1 over-expression is not dependent on transcription, should be part of the article and not part of the supplementary data. Based on these data the authors claim that ATM deficiency increases the stability of lamin B1. It is necessary that the authors show the mechanism by which ATM deficiency stabilizes lamin B1. Is it reduced proteolysis? Are the ubiquitin system or lysosomes involved? Further molecular details are needed to delineate this phenomenon.

We added the QPCR data to Figure 6.

To address the mechanism of lamin B1 degradation, we analyzed the impact of proteasome or lysosome inhibition (using MG132 or leupeptin treatment) on lamin B1 levels. Treatment with the proteasome inhibitor MG132 (but not leupeptin) increased the levels of lamin B1 in wild-type cells, suggesting that at least part of the lamin B1 turnover regulation involved the proteosomal pathway (Figure S5 C). We also showed the physical interaction between lamin B1 and p38 MAPK and *in vitro* phosphorylation of lamin B1 by p38 MAPK (Figure 7).

**5)** The authors used anisomycin was used as a p38 MAPK activator. However, anisomycin is also a very potent protein synthesis inhibitor. In this respect, this experiment is a replication of the cycloheximide experiment.

Both wild-type and A-T cells were comparatively exposed to cycloheximide, which revealed a strong difference between wild-type and A-T cells. Of note, treatment of wild-type cells with cycloheximide decreased lamin B1 levels, while anisomycin increases lamin B1 levels. These data indicate the difference in response between the two treatments and suggest that they act on different pathways. In a parallel experiment, inhibition of p38 MAPK by SB203580 or siRNAs lead to a decrease in lamin B1 levels, suggesting that p38 MAPK is responsible for the increase in lamin B1.

**6)** Will increased oxidative stress in the absence of lamin B1 cause senescence?

We cannot perform this experiment because a decrease in lamin B1 levels leads to nuclear morphology alterations and senescence in wild-type cells (unpublished observation from

our laboratory and as suggested by data in LMNB1 $\Delta/\Delta$ , Vergnes et al., 2004, Proc Natl Acad Sci USA, 101:10428-433). Moreover, simultaneous H<sub>2</sub>O<sub>2</sub> treatment and siRNA transfection are toxic for consistent analysis.

**7)** Figure 2: I would suggest that the authors present higher magnification photographs of the altered shape nuclei and better describe the criteria used to determine abnormal nuclear shape. This also applicable to Figure 4.

As suggested by this referee, we have added representative images (higher magnification) of nuclear changes in Figure 2.

As already discussed above in the answers to comments by the editor and referees 1 and 2, we used the Cell Profiler software to measure the circularity of the nuclei. We described the module used in the Material and Methods sections and page 7 of the manuscript. We used the software to analyze nuclear shape alterations in the different situations described in the paper (Figures 2, 4, 5 (Fig. 4 of previous version), 8, and S8).

**8)** The authors should show the effects of NAC on lamin B1 expression in WT cells.

These data are now shown in Figure S4 and discussed on page 10. NAC treatment decreases lamin B1 levels in WT cells, reflecting the impact of the endogenous oxidative stress.

Minor points:

1. For the general reader please provide the information that BSO causes oxidative stress through the inhibition of GSH synthesis.
2. Page 15, third line: DRR should be DDR.
3. Page 17, line 13: Anti-p(S1982)-ATM should be anti-p(S1981)-ATM.

We have added these corrections.



Thank you for submitting your revised manuscript for consideration by The EMBO Journal. After some delay owed to restricted availability of the original reviewers, we have now obtained re-reviews from all three of them. I am pleased to inform you that all of them consider the study, in particular with regard to the major concerns, substantially improved, and now in principle suitable for publication in the journal. Referees 1 and 2 still list several specific points that would need to be cleared up - for the most part these should however not necessarily require further experimentation, but may be addressable by text modifications and changes to the (data) presentation or interpretation. There is only one potentially more significant technical issue, raised by referee 2 in his/her last point, which I would like to ask you to seriously consider and hopefully clarify, if needed with additional control data.

I am therefore returning the study to you once more for an ultimate round of re-revision, hoping you will be able to resubmit your final version soon. When doing so, please again include a brief letter of response to the new referee comments; and please make sure to upload the main figures (1-9) as individual files, i.e. one file per figure.

Once carefully revised, please re-submit the final version as usual through our website. I am hoping that following adequate re-revision, we should then be able to proceed with eventual acceptance and publication of the study. Should you have any further questions in this regard, please do not hesitate to contact me.

Sincerely,

Editor  
The EMBO Journal

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#### REFeree REPORTS:

##### Referee #1 (Remarks to the Author):

Lamins have been shown to play an important role in many cellular processes. In this manuscript Barascu et al describe a new role for lamin B1 in premature senescence in A-T cells and in general. The authors show that in A-T cells (lymphoblasts and fibroblasts), which exhibit nuclear shape alterations (NSA) and undergo premature senescence, lamin B1 levels, but not lamin A,C and B2, are increased compared to cells from healthy individuals. The increased lamin B1 levels are very likely directly responsible for NSA as well as for premature senescence, as over-expression of lamin B1 in wild-type fibroblasts leads to similar phenotypes, while RNAi of lamin B1 in A-T cells results in a decrease in NSA and premature senescence. The authors further showed that the increase in lamin B1 results from the increased oxidative stress occurring in A-T cells. To analyze if lamin B1 is upregulated on the transcriptional level, Barascu et al performed qPCR. Surprisingly they found a decrease of lamin B1 mRNA levels. Further analyzes revealed that lamin B1 protein levels are stabilized in A-T cells by p38 MAPK, which is activated due to endogenous oxidative stress. In conclusion, p38 MAPK is shown to phosphorylate lamin B1 in vitro and inhibition of p38 MAPK in A-T cells leads to a decrease in lamin B1 levels, NSA and premature senescence.

This is an interesting work not only for people working on nuclear lamins or ATM but for a more general scientific community, as the authors provide insight into the mechanisms how oxidative stress in general might lead to senescence. Therefore I recommend it for publication in EMBO J with minor revisions, which can possibly be addressed without any additional experiments.

Specific comments:

Figure 2B: The lamin B1 immunofluorescence staining does not correlate with the lamin B1 signal in the Western Blot. The authors should comment on this.

Figure 3A: In the text it says that 80% of A-T cells are SA- -gal positive at passage 22, while in the figure it says 73%. Please make it consistent.

Figure 3C: I wonder why the HP1 staining is so weak in cells transfected with the control plasmid? It would be of benefit if the authors could also add a lamin B1 staining to show a direct correlation of increased lamin B1 and senescence associated heterochromatin foci.

Figure S3A: Right panel labeled control: what does it show? Why is there a lamin B1 double band? What is the strong band visible above the Chk1 and P-Chk1 band?

Figure S3B: The authors claim that lamin B1 overexpression leads to NSA. However, the nuclei the authors are showing in the right panel (LMNB1) does not show NSA. Therefore I suggest that the authors also show a lamin B1 staining.

Figure 4B: The right panel (Merged) is not necessary, as the DAPI staining is anyway not well visible. Therefore I recommend that the authors either make a better merge of DAPI and lamin B1 or remove this panel.

Figure 5B: The increase of lamin B1 in A-T cells compared to wt cells is much higher than in all other figures. The authors should comment on this.

Figure 5B and C: The lamin B1 levels shown in the Western blot are lower in the wt cells treated with H<sub>2</sub>O<sub>2</sub> compared to the A-T cells treated with NAC. However, the authors claim that in the latter less NSA are observed. Similarly, over two times more lamin B1 in untreated A-T cells compared to H<sub>2</sub>O<sub>2</sub> treated wt cells results in the same percentage of cells showing NSA. This is in contradiction to the model that higher levels of lamin B1 lead to more NSA. The authors should comment on this.

Figure 7C: The authors should show a control IP (eg. just beads), which they have likely done, to proof the specificity of the lamin B1-p38MAPK interaction. In addition, the lamin B1 Co-IP in M&M is poorly described. I am wondering how lamin B1 is soluble in a buffer containing only 150 mM salt? The authors should provide a more detailed description of the Co-IP, as this is an essential experiment of the paper.

Figure 7D: I am wondering why there is no P-p38 MAPK detectable in the lamin B1 immunoprecipitates? Also the description of the in vitro phosphorylation experiment is poor in M&M. Where again lamin B1 transfected cells used?

Figure S9A: Why are the p38MAPK levels much lower in the GM03348 cells compared to the GM03652 cells, although the P-p38MAPK levels are the same in both cells lines?

Referee #2 (Remarks to the Author):

The authors have put substantial effort into addressing the points of the referees, and with a few exceptions, they have done a good job. All of my suggestions were answered to sufficient degree. I note the few exceptions below:

Reviewer #3, point 3, asked for "additional data regarding the mechanism by which lamin B1 overexpression inhibits cell division". The authors responded with a BrdU incorporation assay and a western blot of cyclin A. While these assays confirm a lack of cell division, they are not mechanistic, as the term is generally understood. Granted, "additional mechanism" is always a difficult request, but it should be noted that the response did not address the point.

I don't think the negative feedback argument discussed on page 11 (to explain why the protein levels are increased while the mRNA levels are decreased) is particularly compelling. Negative feedback loops are generally short-term phenomena that persist only until homeostasis is regained. That is, discrepancies between mRNA and protein level like the one observed here only persist for a short period. The authors examine unchallenged A-T cells, so must invoke a perpetual negative feedback loop that causes a decrease in the mRNA level at the same time as an increase in the protein level (due to a stability shift). This is a possible, but in my opinion implausible, explanation. Given this strange relationship between mRNA and protein, it more mRNA analysis might have been appropriate.

The authors have, via immunofluorescence 1) measured circularity of individual nuclei and 2) measured the lamin B1 intensity of individual nuclei, presumably in the same samples. Because only a fraction of cells had abnormal nuclei, it would be nice to see these two measurements plotted on orthogonal axes. One would expect there to be a correlation between lamin B1 increase and abnormal nuclear shape.

Figure 7A: why are the anisomycin bands shifted to higher MWs?

In the figure legend of S5, the first line mistakenly refers the reader to Figure 7B - I believe it should be 6B

Figure S6A and S6C: total p38 should be shown, in case total levels vary substantially between cells types, as they do (for example) in Figure S9A.

Figures S7 is oddly constructed: why are the H2O2 treated samples without SB203580 (part A) not combined on the same graphs and blots as the SB203580-treated samples (part B)? The lamin B1 levels should be compared to the same control, i.e. DMSO treated cells - not DMSO-treated cells in part (A) and SB23580-treated cells in part (B). I mention this primarily because it seems like the westerns in parts (A) and (B) are from similar, if not the same, blots.

\*\*One last, potentially important, point: I noticed up re-reading that the authors use a lysis buffer containing 8M urea, followed by an extremely high-speed centrifugation to pellet insoluble material, before analyzing samples via western blot. While this would normally not be an issue, lamin solubility can be tricky, and 8M urea followed by high-speed centrifugation is, in fact, part of the standard protocol to precipitate lamins. Thus, it is possible that the authors are pelleting and discarding the majority of their lamin protein, and thus any changes via western blot could be due to solubility changes rather than actual changes in protein level. Lamins change their solubility in response to nuclear reorganization, and nuclear reorganization is certainly part of the senescence phenotype. The authors may want to verify their findings using a lysis recipe that includes SDS. Apologies to the authors for this late-stage suggestion, but I did not notice it the first time.

Referee #3 (Remarks to the Author):

I recommend to accept the paper for publication in EMBO J. The authors addressed all my questions and concerns. It is indeed a very interesting and well written paper. I enjoyed reading it. The paper present novel and important findings.

Dear Editor

Thank you for giving us the opportunity to re-submit a final version of the manuscript: "**ATM-alternative senescence pathway induced by lamin B1 accumulation through p38 MAPK activation in response to oxidative stress**"

We have addressed referees' comments and we hope that the manuscript is now acceptable for publication in *EMBO Journal*.

**ANSWERS to REVIEWERS' comments.**

**Referee#1 (Remarks to the Author):**

**1)** "Figure 2B : Figure 2B: The lamin B1 immunofluorescence staining does not correlate with the lamin B1 signal in the Western Blot. The authors should comment on this."

It is difficult to compare the increase of lamin B1 monitored by two different procedures (WB vs. immunofluorescence). In the present revised version, we show a more representative WB which reflects the average increase of the lamin B1 obtained following surexpression.

**2)** "Figure 3A: In the text it says that 80% of A-T cells are SA- $\beta$ -gal positive at passage 22, while in the figure it says 73%. Please make it consistent."

We thank the referee to point out this discrepancy. The data are now corrected.

**3)** "Figure 3C: I wonder why the HP1 staining is so weak in cells transfected with the control plasmid? It would be of benefit if the authors could also add a lamin B1 staining to show a direct correlation of increased lamin B1 and senescence associated heterochromatin foci."

The low level of the HP1 signal in wild-type cells is due to the setting acquisition parameters. Indeed, we fixed the setting parameters to avoid saturation of HP1 signal in the condition of lamin B1 overexpression. As we always apply the same parameters in all conditions, we obtain a weak signal in the control cells.

We can see the direct correlation between increased lamin B1 and SAHF in figure 2B where the DAPI is now presented in grey level (which allows a clearer visualisation of the SAHF).

**4)** "Figure S3A: Right panel labeled control: what does it show? Why is there a lamin B1 double band? What is the strong band visible above the Chk1 and P-Chk1 band?"

The control for positive detection of proteins by WB is HeLa cells. The HeLa cell line has lost checkpoints control and as a consequence accumulates spontaneous DNA damages resulting in constitutive activation of the DNA damage response. Thus, it is a convenient positive control allowing to test by immunodetection of both P-Chk1 and P-Chk2 proteins, which normally require different stress to be activated. The double band of lamin B1 likely reflects post-translational modifications. The visible band above the Chk1 and P-Chk1 corresponds to the previous immunodetection on the same membrane of the Chk2 protein, which is highly expressed in the control HeLa cells and persists in spite of the stripping step.

**5)** "Figure S3B: The authors claim that lamin B1 overexpression leads to NSA. However, the nuclei the authors are showing in the right panel (LMNB1) does not show NSA. Therefore I suggest that the authors also show a lamin B1 staining."

Unfortunately, immunostaining of the lamin B1 was not performed in these sets of experiments. However, it should be pointed out that transfection with the lamin B1 expression plasmid strongly increased the frequency of cells with NSA and SAHF (Figures 2B and 3C).

In the revised version we present a novel representative figure of a nuclei with NSA and SAHF.

**6)** "Figure 4B: The right panel (Merged) is not necessary, as the DAPI staining is anyway not well visible. Therefore I recommend that the authors either make a better merge of DAPI and lamin B1 or remove this panel."

We do agree, the right panel (merge) is not necessary; we removed it.

**7)** "Figure 5B: The increase of lamin B1 in A-T cells compared to wt cells is much higher than in all other figures. The authors should comment on this."

When comparing this figure to the figure 1A, the increase of lamin B1 in A-T cells compared to WT does not seem so different.

**8)** "Figure 5B and C: The lamin B1 levels shown in the Western blot are lower in the wt cells treated with H2O2 compared to the A-T cells treated with NAC. However, the authors claim that in the latter less NSA are observed. Similarly, over two times more lamin B1 in untreated A-T cells compared to H2O2 treated wt cells results in the same percentage of cells showing NSA. This is in contradiction to the model that higher levels of lamin B1 lead to more NSA. The authors should comment on this."

There was a misunderstanding, the Figure 5B referred to lymphoblasts and figure 5C to fibroblasts. Therefore results of nuclei circularity obtained on primary fibroblasts and presented on 5C must be compared with WB of the figure 5A obtained also in primary fibroblasts. To clarify this point and facilitate the reading, we switched now figures 5A and 5B, to present the lymphoblasts results in 5A and then results obtained with fibroblasts in 5B and C.

**9)** "Figure 7C: The authors should show a control IP (eg. just beads), which they have likely done, to proof the specificity of the lamin B1-p38MAPK interaction. In addition, the lamin B1 Co-IP in M&M is poorly described. I am wondering how lamin B1 is soluble in a buffer containing only 150 mM salt? The authors should provide a more detailed description of the Co-IP, as this is an essential experiment of the paper."

We changed the WB presented in 7C by images of another experiment where appears the negative control (beads alone).

We improved the M&M. First, we have detailed the lysis buffer composition (NaCl 150 mM, NP40 1%, EDTA 1 mM, Tris 25mM pH 7,5). We specified that to improve the efficiency of protein extraction, we repeated mechanical disruption by passing the lysate 10 times through a needle attached to a 0.3 ml syringe.

Importantly, the Co-IP results are confirmed with another experimental approach: the PLA. This experiment allows the *in situ* detection of the interaction between endogenous lamin B1 and P-p38 MAPK proteins. Taken together, the results of these two experiments allowed us to conclude that these two proteins physically interact.

**10)** "Figure 7D: I am wondering why there is no P-p38 MAPK detectable in the lamin B1 immunoprecipitates? Also the description of the *in vitro* phosphorylation experiment is poor in M&M. Where again lamin B1 transfected cells used?"

In kinase assay only proteins that are phosphorylated and thus have incorporated  $P^{32}$  can be detected. To perform this assay, we have added 1 $\mu$ g of recombinant alpha-p38 MAPK and 1 $\mu$ g of recombinant MKK3, 5  $\mu$ M ATP and 5 $\mu$ Ci ( $\gamma$ - $^{32}$ P)ATP on ATF2 substrate (positive control, on first line) or lamin B1 immunoprecipitated (on lines 3 and 4). We can detect p38 MAPK in the line 1 because: first, p38 MAPK is phosphorylated and activated by MKK3 and second we loaded the totality of the sample. In the line 3 and 4, it's difficult to detect P-p38 MAPK because we loaded only part of the samples to avoid the saturation of phosphorylated lamin B1 signal. In addition, it is also important to note that for this experiment using lamin B1 immunoprecipitation we used the RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS) that is suitable for immunoprecipitation but not recommended for co-immunoprecipitation (as it is partly denaturing due to the ionic detergents SDS and sodium deoxycholate). As request by the referee, we improved the description of this experiment in M&M.

**11)** "Figure S9A: Why are the p38MAPK levels much lower in the GM03348 cells compared to the GM03652 cells, although the P-p38MAPK levels are the same in both cells lines?"

The main conclusion of the presented blot is the negative effect of lamin B1 overexpression on P-p38MAPK levels and we can see also that this effect is not due to the decreased of p38 MAPK total level. We think that the difference of p38 MAPK total level observed between both primary fibroblasts in the figure S9A was due to a problem on the detection of total p38 MAPK in this particular experiment. To avoid any confusion we present now another blot, with a better detection of total p38MAPK in both cells lines; this confirms our conclusions.

#### **Referee #2 (Remarks to the Author):**

The authors have put substantial effort into addressing the points of the referees, and with a few exceptions, they have done a good job. All of my suggestions were answered to sufficient degree. I note the few exceptions below:

**1)** "Reviewer #3, point 3, asked for "additional data regarding the mechanism by which lamin B1 overexpression inhibits cell division". The authors responded with a BrdU incorporation assay and a western blot of cyclin A. While these assays confirm a lack of cell division, they are not mechanistic, as the term is generally understood. Granted, "additional mechanism" is always a difficult request, but it should be noted that the response did not address the point."

As done for most of the new senescence model, we have documented on the growth arrest using the classical BrdU assay and detection of protein associated with cell cycle progression. Of course we could have pursued on this issue and address more mechanistic questions, but we have already address several important questions in the present paper. The mechanistic of the overexpressed lamin B1 induced arrest is indeed an important question on its own, which deserved to be addressed in full detailed and dedicated manuscript.

**2)** "I don't think the negative feedback argument discussed on page 11 (to explain why the protein levels are increased while the mRNA levels are decreased) is particularly compelling. Negative feedback loops are generally short-term phenomena that persist only until homeostasis is regained. That is, discrepancies between mRNA and protein level like the one observed here only persist for a short period. The authors examine unchallenged A-T cells, so must invoke a perpetual negative feedback loop that causes a decrease in the mRNA level at the same time as an increase in the protein level (due to a stability shift). This is a possible, but in my opinion implausible, explanation. Given this strange relationship between mRNA and protein, it more mRNA analysis might have been appropriate."

In our final model (Fig. 9), in normal physiological conditions, the increase of lamin B1 is part of the response to OS and the level of lamin B1 should come back to the steady state when the problem (OS) is fixed; the overexpression of lamin B1 should thus be transient and a negative feedback regulation should operate (consistently with the referee's remark). However, AT cells are not physiological but pathological conditions and cell homeostasis might be strongly altered: the OS is constitutive and the response should also be constitutive; however since lamin B1 overexpression arrests cell proliferation, some, at least partial, compensation should exist to allow AT cells to proliferate. The low level of lamin B1 mRNA might reflect this partial compensation; without this mRNA decrease the level of lamin B1 should be even much more higher and might be not compatible with proliferation of AT cells.

**3)** "The authors have, via immunofluorescence 1) measured circularity of individual nuclei and 2) measured the lamin B1 intensity of individual nuclei, presumably in the same samples. Because only a fraction of cells had abnormal nuclei, it would be nice to see these two measurements plotted on orthogonal axes. One would expect there to be a correlation between lamin B1 increase and abnormal nuclear shape."

This is a very good suggestion and we do present now a figure (Figure S2B) with both parameters.

**4)** "Figure 7A: why are the anisomycin bands shifted to higher MWs?"

The shift is probably due to a migration problem. This is specific to this experiment and doesn't change the main message.

**5)** "In the figure legend of S5, the first line mistakenly refers the reader to Figure 7B - I believe it should be 6B."

The mistake is now corrected.

**6)** "Figure S6A and S6C: total p38 should be shown, in case total levels vary substantially between cells types, as they do (for example) in Figure S9A."

The difference on p38 MAPK total level observed between the two primary fibroblasts presented in the figure S9A is likely due to a problem on the detection of p38 MAPK total in this particular experiment (see answer 11 to referee 1). Furthermore, it's important to note that the level of p38 MAPK remained unchanged in the conditions compared (LMNB1 vs control plasmid transfection). Nevertheless, to avoid any confusion we present other one blot with a better detection of p38 MAPK. Indeed, we did not observe any variation in the total level of p38 MAPK between the analysed cell types (see new Figure S6A).

**7)** "Figures S7 is oddly constructed: why are the H<sub>2</sub>O<sub>2</sub> treated samples without SB203580 (part A) not combined on the same graphs and blots as the SB203580-treated samples (part B)? The lamin B1 levels should be compared to the same control, i.e. DMSO treated cells - not DMSO-treated cells in part (A) and SB203580-treated cells in part (B). I mention this primarily because it seems like the westerns in parts (A) and (B) are from similar, if not the same, blots."

In upper panel, we have treated cells with DMSO to exclude a potential effect of DMSO itself and to place control cells in comparable conditions than cells treated with SB203580, which is diluted in DMSO. In our view, the right control for H<sub>2</sub>O<sub>2</sub> treated cells without SB203580 is the DMSO treated cells, and the right control for H<sub>2</sub>O<sub>2</sub> treated samples with SB203580, is the SB203580 treated cells. This is the reasons why we presented the results in such a way.

**8)** "\*\*One last, potentially important, point: I noticed up re-reading that the authors use a lysis buffer containing 8M urea, followed by an extremely high-speed centrifugation to pellet insoluble material, before analyzing samples via western blot. While this would normally not be an issue, lamin solubility can be tricky, and 8M urea followed by high-speed centrifugation is, in fact, part of the standard protocol to precipitate lamins. Thus, it is possible that the authors are pelleting and discarding the majority of their lamin protein, and thus any changes via western blot could be due to solubility changes rather than actual changes in protein level. Lamins change their solubility in response to nuclear reorganization, and nuclear reorganization is certainly part of the senescence phenotype. The authors may want to verify their findings using a lysis recipe that includes SDS. Apologies to the authors for this late-stage suggestion, but I did not notice it the first time."

In this new revised manuscript, we improved the M&M concerning the lamin B1 extraction.

We choose lysis buffer containing 8M urea, because high urea concentration is commonly used to extract or solubilize lamins (e.g, Georgatos and Blobel, JCB, 1987; Gerace et al, JCB 2001; Otto et al, Eur. J. Biochem, 2001; for review Krohne, Methods in Cell Biol, 2001).

Nevertheless, to comply with referee request, we show below the lamin B1 detection in extracts prepared with SDS-laemmli buffer. The increase of lamin B1 level is confirmed showing that is not due to extraction method.

Moreover, this conclusion is supported by immunofluorescence studies all along the manuscript and experiments of protein stability, which confirm our conclusions. In addition, the fact that the rescue of lamin B1 level by siRNA, rescues senescence and nuclear shape, confirms again that the difference in lamin B1 level is actually responsible for both NSA and SAHF.

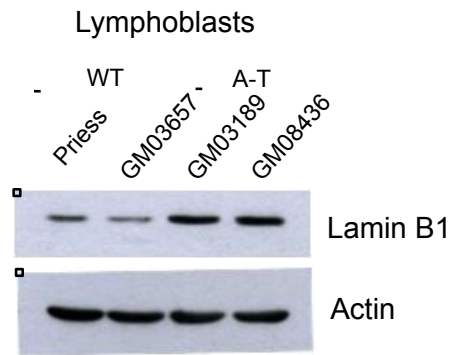


Figure: Western blot analysis of extracts from wild-type (Priess and GM03657) and A-T (GM03189 and GM08436) lymphoblasts. An increase of lamin B1 level was confirmed in A-T compared to the WT cells when extracts were prepared with other lysis buffer: the Laemmli buffer. Briefly, proteins extracts were obtained by adding SDS-Laemmli buffer with phosphatases and proteases inhibitors on cells. Following mechanical disruption by passing the lysate 10 times through a needle attached to a 0.3 ml syringe, the lysate was sonicated. Following denaturation at 95°C for 5 min, an equal volume of protein extract was loaded on gel.



Acceptance letter

19 December 2011

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Thank you for submitting your re-revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Yours sincerely,  
Editor  
The EMBO Journal