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Regulation of genomic stability and senescence by the AMPK-related kinase ARK5

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1st Editorial Decision 29 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, they all find the link of ARK5 and LATS1 to replicative senescence you identified potentially interesting and therefore in principle worth of reporting in The EMBO Journal. All of them nevertheless also bring up a number of substantive (and partially recurring) issues that would need to be satisfactorily addressed or clarified before publication may be warranted.

Should you be able to address these points, we should be happy to consider a revised manuscript for publication. I would therefore like to invite you to prepare such a revised version, taking into account the various comments and suggestions raised by all three referees. Please let me add that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore important to adequately answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Humbert et al employ a shRNA retroviral library in order to identify components that regulate cellular senescence of human cells. Through this screen, they identify ARK5 as a promoter for this intrinsic process and normal senescing cells express high amounts of ARK5. Over-expression of ARK5 alone promotes premature senescence, suggesting that senescence is indeed modulated by ARK5. The duality of ARK5 on senescence is one that is novel and interesting. The authors go on to identify LATS1 as a key target of ARK5 mediated kinase activity, where phosphorylation seems to promote the subsequent down regulation of LATS1, leading to senescence. The mechanism mediating senescence due to this phosphorylation is unclear, however. Does phosphorylation destabilize LATS1 and promote its degradation? One could use proteosome inhibitors to expand on this portion of the manuscript. Overall, the story is quite cohesive about how ARK5 mediates senescence.

ARK5's mechanism mediating senescence is unclear. The authors suggest a linkage that ARK5 expression promotes genomic instability and ultimately senescence. The reports of premature senescence in cells due to genomic instability are somewhat different than the mechanism proposed and appear to have some links to an elevated p53 and Rb response, which is not evident in this model. These are important points to bring out in the discussion, such as differences in cell types, organism, etc. The supplementary figure discussing this issue warrants more attention than it is given in the original text. Also, the figures addressing this issue demonstrate that the cells have an aberrant chromosomal content, but whether they are unstable is not supported. Are the authors certain that the cells just haven't undergone a replication without cytokinesis? I don't think the data is conclusive enough in its current form to say that ARK5 promotes genomic instability. Are these cells re-entering the cell cycle and acquiring additional chromosomal changes? This is not clear.

Major points:
1. Why do ARK5 shRNA cells ultimately senescence? Is the explanation as simple as the down-regulation of ARK5 ceases at later passages in these cells? The growth curves suggest that there is an ultimate endpoint to their proliferative capacity, however there is no follow up to this finding. If LATS1 is the key target, is it being phosphorylated and destabilized by one of the other kinases in the family?
2. If ARK5 is a master regulator of senescence, does a complete knockout of ARK5 in cells result in spontaneous immortalization? The ARK5 kinase dead infected cells grow better than control cells. If followed for more passages, does expression of the kinase dead version of ARK5 result in immortalization?
3. If cells over-express ARK5 become senescent, is there any way to remove this over-expression and assess if the cells are able to re-enter the cell cycle? This would be an interesting experiment to see if the cells are irreversibly senescent.
4. Figure 7C bottom left panel: Is it really probed for ARK5 or is that a FLAG antibody blot? It is confusing as to why NUAK2 would lead to a higher level of ARK5 if the figure is correct. Is NUAK2 dependent on ARK5 in some way?

Minor points:
1. Page 2, line 12 of the abstract should read "plays" not "play".
2. Page 5, last line should be Q-PCR or qRT-PCR.
3. This reviewer does not like the usage of the term "lifespan" when it comes to senescent cells in culture. By definition, senescent cells in vitro are not dead; in fact they are quite alive. Organismal lifespan has a clear end point, death. I think of cells in the same way. If the authors prefer to use this term, they should be using replicative lifespan in my opinion.
4. Similarly, the usage of young versus old to describe the cells is confusing.

Altogether, this is very interesting piece of work that identifies a novel protein with dual effects on senescence depending on the expression, where the data well support these conclusions. The

connection between genomic instability and senescence may be a little over stated however.

Referee #2 (Remarks to the Author):

In this article, the authors describe the role of ARK5 in regulating the cellular senescence of human fetal fibroblasts. The authors present data showing that ARK knockdown leads to a delay in senescence whereas ARK overexpression accelerates senescence. They provide evidence that Lkb1-mediated activation of ARK is required for its capacity to induce senescence. In addition, they show that ARK5 can phosphoryate Lats1 and reduce expression of Lats1 protein, which may account for the ability of ARK5 to induce senescence.

This is a well-executed study that provides novel insights into the mechanisms of senescence. The data are convincing and support the conclusions of the study.

Points that need addressing are enumerated below:

The paper shows that ARK5 knockdown leads to a delay in senescence of about 8 population doublings. The authors should clarify what happens at the later passages. Do the cells ultimately completely undergo growth arrest and show all the typical features of senescence (SA-betaGal, morphological changes, SAHF, polynucleate cells, etc)? This data should be presented.

The authors show that E7 does not rescue premature senescence induced by ARK5 overexpression. They should confirm that their E7 vector produced the expected extension of replicative capacity in the near-senescent WI-38 cells. It would also be helpful to explore whether E6 or telomerase overexpression affect the premature senescence induced by ARK5.

The authors should confirm that kinase-dead ARK5 does not affect LATS1 levels. They should also confirm that ARK5 does not affect LATS1 transcript levels. Finally they should address whether alterations in ARK5 affect LATS1 protein stability.

The significance of of LATS1 downstream of ARK5 needs to be explored more fully. Does LATS1 kinase-dead overcome the senescence bypass provoked by ARK5 knockdown?
Does LATS1 overexpression rescue the premature senescence provoked by ARK5 overexpression?

The manuscript needs to be clearer regarding whether ARK5 is regulating senescence associated with telomere erosion, in comparison to senescence due to culture conditions. My understanding is that the initial barrier encounter in WI-38 cells involves a stress response to oxidative stress that cannot be fully rescued by telomerase. Throughout the manuscript, the issue needs to be clarified for the reader.

Western blots of ARK5 knockdown in WI-38 cells should be presented in Figure 1.

Representative images of SA-beta-Gal and SAHF should be shown in Fig 1D, E and Fig 2E, F.

The manuscript shows that ARK5-overexpressing have increased numbers of polynucleated cells and correspondingly increased numbers of chromosomes. Chromosome numbers in Fig 5D should be quantitated.

It would be helpful to have additional information on the initial screen. How many clones were screened? How many shRNA's were found to rescue senescence.

The discussion is very superficial and does not provide much context for this work or address its implications.

Referee #3 (Remarks to the Author):

EMBO J-2009-71492

"Regulation of genomic stability and senescence by the AMPK-related kinase ARK5" by Humbert et al.

The regulation of cellular senescence is intensely interesting as it is associated with both tumor suppression and aging. However the molecular mechanisms involved are not fully understood as yet. In this paper, Humbert and co-workers identified ARK5 as a key regulator of cellular senescence through a loss of function screen using a retroviral shRNA library. Interestingly, the authors found that elevated expression of ARK5 facilitates phosphorylation of LATS1 at Ser464 and thereby causing reduction of LATS1 levels and consequent a cytokinetic block in senescent cells. These finding are novel and provide a new insight into how senescence cell cycle arrest is enforced.

In general, the experiments are well done and data are for the most part solid. I have several issues, however:

(1) The authors have nicely shown that the ectopic expression of ARK5 causes reduction of LATS1 levels through phosphorylation of LATS1 at Ser 464. However, it is unclear how this happens. Takahashi et al (2006) reported that the reduction of WARTS/LATS1 level was attenuated by the addition of MG132, a proteasome inhibitor, in senescent cells, suggesting that LATS1 is destabilized by the proteasome dependent protein degradation machinery during cellular senescence. It is therefore important to examine whether this is also the case in ARK5 induced LATS1 reduction.

(2) Along these lines, although Takahashi et al (2006) failed to prevent reduction of LATS1 level by substitution of Ser 464 to Ala (see Supplementary information 3b), the same substitution mutant (S464A) is resistant to ARK5-induced reduction of LATS1 in this manuscript (Fig.7F). Because Takahashi et al (2006) used SVts8 cells, a conditionally immortalized human fibroblasts, and Humbert (this paper) used 293 cells, it is possible that these seemingly contradictory data are, at least partly, due to the difference of the cellular contexts in these cell lines. It is therefore important to examine whether the S464A mutant is resistant to degradation in senescent human diploid fibroblasts rather than in established cell lines. I realize this issue presents technical challenge that may be formidable, but no doubt this is very important.

(3) Since oncogenic Ras expression is known to induce senescence like cell cycle arrest, it is interesting to know whether ARK5 expression is also induced in Ras-induced senescence. It is also interesting to know whether NUAK2 expression is increased in both replicative- and Ras-induced senescence.

(4) In Fig.4D, ectopic expression of K84A significantly enhanced cellular proliferation in HDFs. Is this because K84A acts as a dominant negative against endogenous ARK5?

(5) The quality of Fig.S3 is too poor. The authors should replace this figure with a higher quality photo.

1st Revision - authors' response 28 September 2009

Please find enclosed a point-by-point response to the referees' comments and a list of the incorporated changes to our paper EMBOJ-2009-71492 (Regulation of genomic stability and senescence by the AMPK-related kinase NUAK1). We now feel that we are able to address the issues raised during the review process.
To respect the official nomenclature we have replaced ARK5 by NUAK1 in the manuscript.

Referee #1
General comments:

-Does phosphorylation destabilize LATS1 and promote its degradation? One could use proteosome inhibitors to expand on this portion of the manuscript.

We have tested the effect of the MG-132 proteasome inhibitor. We did not observe an impact of MG-132 over LATS1 level suggesting that NUAK1 was not decreasing the levels of LATS1 through the proteasome machinery. We have added these results as Supplemental Figure S12 and have added comments in the discussion section p14.

-The reports of premature senescence in cells due to genomic instability are somewhat different than the mechanism proposed and appear to have some links to an elevated p53 and Rb response, which is not evident in this model. These are important points to bring out in the discussion, such as differences in cell types, organism, etc. The supplementary figure discussing this issue warrants more attention than it is given in the original text.

Indeed, one might expect that senescence due to genomic instability results in elevated p53 and Rb pathways. As explained in the manuscript, the Rb pathway is modified by NUAK1 but "the inhibition of the Rb pathway by E7 was not sufficient to revert NUAK1-induced senescence. The modification observed on Rb is thus insufficient to explain NUAK1 effect and could thus be only a mark of the proliferative state." (p8).
We did not observe an increase or decrease of the p53 activity when we manipulate NUAK1 in WI-38 cells. We have added data showing that NUAK1 was still inducing senescence of E6-expressing cells confirming that NUAK1 was acting independently of p53 in this model (Figure S3 and comments added on p8 and p12).
The reason why genomic instability in our models did not result in p53 activation could be that LATS1 inactivation results in p53 inactivation as suggested previously (Iida et al, Oncogene 2004).

-Also, the figures addressing this issue demonstrate that the cells have an aberrant chromosomal content, but whether they are unstable is not supported. Are the authors certain that the cells just haven't undergone a replication without cytokinesis? I don't think the data is conclusive enough in its current form to say that ARK5 promotes genomic instability. Are these cells re-entering the cell cycle and acquiring additional chromosomal changes? This is not clear.

The idea is that the aberrant chromosomal content might cause senescence. So it is probable that when the cells reached the senescent state (so do not further divide) they are not further "unstable", but still they should at some points have some genomic instability to get such an aberrant DNA content at senescence. The heterogeneity of the DNA content (between 2n to 180 chromosomes) observed per cells (see results in figure 5) suggests that the cells do not enter the senescent state in a homogenous manner. So yes probably some cells rentered the cell cycle and acquired additional chromosomal changes before entering in the senescent state whereas others did not.
Nevertheless if the referees view we overstated our results by using "genomic instability" we could instead use a term like "aberrant DNA content".

Major points:

1.Why do ARK5 shRNA cells ultimately senescence? Is the explanation as simple as the down-regulation of ARK5 ceases at later passages in these cells? The growth curves suggest that there is an ultimate endpoint to their proliferative capacity, however there is no follow up to this finding. If LATS1 is the key target, is it being phosphorylated and destabilized by one of the other kinases in the family?

Replicative senescence of human fibroblasts is mostly due to telomere shortening. Thus, expression of hTert, the catalytic subunit of the telomerase, immortalizes such cells (see for example Augert et al, EMBO R, 2008). Expression of hTert in WI38 cells knock down for NUAK1 resulted in cellular immortalisation showing that shNUAK1-expressing cells senesce due to telomere shortening. We have now added these results as Figure S1 and comments are added on p5.

2.If ARK5 is a master regulator of senescence, does a complete knockout of ARK5 in cells result in spontaneous immortalization? The ARK5 kinase dead infected cells grow better than control cells. If

followed for more passages, does expression of the kinase dead version of ARK5 result in immortalization?

We did not observe any immortalisation of the cells by the shRNA directed against NUAK1 or by using the NUAK1 kinase dead mutant. In the same manner as the shNUAK1-expressing cells, the NUAK1 kinase dead expressing cells are immortalised by the expression of hTert. These results were added as Figure S2 and comments are added on p8.

3. If cells over-express ARK5 become senescent, is there any way to remove this over-expression and assess if the cells are able to re-enter the cell cycle? This would be an interesting experiment to see if the cells are irreversibly senescent.

To address this issue, we have infected cells with an NUAK1-encoding vector and puromycin-selected for 3 days. At day 4, we re-infected the cells with an shRNA NUAK1-encoding vector and maintain a puromycin selection. Although the infection efficiency was quite low (about 20%), we were expecting to see emerging clones if the effect of NUAK1 at day 5 was reversible. Results were added as Figure S11 and comments are added on p14.

4. Figure 7C bottom left panel: Is it really probed for ARK5 or is that a FLAG antibody blot? It is confusing as to why NUAK2 would lead to a higher level of ARK5 if the figure is correct. Is NUAK2 dependent on ARK5 in some way?

Indeed, it was probed with an anti-Flag antibody. We apologise for this labelling mistake.

Minor points:

1.Page 2, line 12 of the abstract should read "plays" not "play".
This mistake was corrected.

2.Page 5, last line should be Q-PCR or qRT-PCR.
We modified by Q-PCR.

3.This reviewer does not like the usage of the term "lifespan" when it comes to senescent cells in culture. By definition, senescent cells in vitro are not dead; in fact they are quite alive. Organismal lifespan has a clear end point, death. I think of cells in the same way. If the authors prefer to use this term, they should be using replicative lifespan in my opinion.
We have added replicative as suggested by the referee all along the manuscript.

4.Similarly, the usage of young versus old to describe the cells is confusing.
We replaced "young" by "early passage or proliferating" and "old" by "senescing or late passage".

Altogether, this is very interesting piece of work that identifies a novel protein with dual effects on senescence depending on the expression, where the data well support these conclusions. The connection between genomic instability and senescence may be a little over stated however.

We have tried to tone down this proposed link. For example in the introduction section we replaced "This screen has revealed that the level of NUAK1 and the resulting level of genomic instability have an impact on the replicative potential of normal human cells." by "This screen has revealed that the level of NUAK1 has an impact on the replicative potential of normal human cells and this might be due to the level of genomic instability".

Referee #2
Points that need addressing are enumerated below:
1.The paper shows that ARK5 knockdown leads to a delay in senescence of about 8 population doublings. The authors should clarify what happens at the later passages. Do the cells ultimately

completely undergo growth arrest and show all the typical features of senescence (SA-betaGal, morphological changes, SAHF, polynucleate cells, etc)? This data should be presented.

We have now added experiments showing that senescing WI38 NUAK1 knock down cells displayed features of senescence: they were SA- -Gal positive, they displayed the typical morphological changes and they were immortalised by hTert expression. These data are presented as Figure S1 and comments are added on p5.

2.The authors show that E7 does not rescue premature senescence induced by ARK5 overexpression. They should confirm that their E7 vector produced the expected extension of replicative capacity in the near-senescent WI-38 cells. It would also be helpful to explore whether E6 or telomerase overexpression affect the premature senescence induced by ARK5.

A growth curve has been included as Figure S3D showing that E7 as well E6 extended the replicative potential of the WI38 cells. Colony assays experiments (Figure S3C) were added to demonstrate that NUAK1 still induces senescence in E6-expressing WI38 cells and in hTert-immortalised WI38 cells.
Comments are added in the results (p8) and in the discussion (p12) section.

3.The authors should confirm that kinase-dead ARK5 does not affect LATS1 levels. They should also confirm that ARK5 does not affect LATS1 transcript levels. Finally they should address whether alterations in ARK5 affect LATS1 protein stability.

-To address this comment and the comment of the referee 3 (point 4), we checked the ability of NUAK1 kinase dead mutant to revert NUAK1 WT effect over LATS1 (Figure S6 and comments are added on p10).
-We have performed RT-PCR analysis on RNA extracted from 293 cells transfected with LATS1 with control or with LATS1 with NUAK1. These results demonstrated that NUAK1 expression did not alter LATS1 mRNA levels (Figure 7C and comments are added on p10).
-NUAK1 did not alter the level of LATS1 mRNA levels. NUAK1 did not alter the levels of LATS1 when the S464 is mutated in A. These both results support a regulation of the LATS1 protein by NUAK1. As explained above (referee 1), the proteasome inhibitor MG-132 did not revert LATS1 decrease by NUAK1 demonstrating that LATS1 is not degraded by the proteasome. These results are presented as Figure S12 and discussed in p14.

4.The significance of of LATS1 downstream of ARK5 needs to be explored more fully. Does LATS1 kinase-dead overcome the senescence bypass provoked by ARK5 knockdown? Does LATS1 overexpression rescue the premature senescence provoked by ARK5 overexpression?
We have stably expressed LATS1 DN mutant in shNUAK1-expressing cells and found

that it is blocking their growth supporting that LATS1 could be downstream of NUAK1. Data were added as Figure S5 and comments are added in p10.
We did not perform the complementary experiment as LATS1 constitutive expression by itself can inhibit cell growth as told in the introduction section "Strong enforcement of genomic stability safeguards such as by LATS1 or BubR1 constitutive expression can even inhibit cell growth (Iida et al, 2004; Shin et al, 2003; Yang et al, 2001)".

5.The manuscript needs to be clearer regarding whether ARK5 is regulating senescence associated with telomere erosion, in comparison to senescence due to culture conditions. My understanding is that the initial barrier encounter in WI-38 cells involves a stress response to oxidative stress that cannot be fully rescued by telomerase. Throughout the manuscript, the issue needs to be clarified for the reader.

All our results strongly supports that NUAK1 is acting independently of telomeres erosion as:
-hTert expression bypassed senescence of shNUAK1-expressing cells (see Figure S1)
-NUAK1 expression induced senescence in hTert-immortalised cells (see Figure S3)
-NUAK1 did not impact the p53 pathway, which is regulated by the telomere erosion.
In our hand hTert expression immortalised WI38 cells (see supplemental figure 1 in Augert et al, EMBO R, 2009). We have now added a new paragraph in the discussion section p12 arguing that

NUAK1 acts independently of the telomere pathway, or downstream through an alternate (p53 independent) pathway.

6.Western blots of ARK5 knockdown in WI-38 cells should be presented in Figure 1.
We have replaced the previous data in 293 cells by data in WI-38 cells. Figure Legend was modified accordingly.

7.Representative images of SA-beta-Gal and SAHF should be shown in Fig 1D, E and Fig 2E, F.
These images have been added.

8.The manuscript shows that ARK5-overexpressing have increased numbers of polynucleated cells and correspondingly increased numbers of chromosomes. Chromosome numbers in Fig 5D should be quantitated.
The chromosome numbers were quantified as asked.

9.It would be helpful to have additional information on the initial screen. How many clones were screened? How many shRNA's were found to rescue senescence.
Detailed concerning the screen has been added by citing the first paper describing the shRNA library we used (Berns et al, 2004) and by adding comments about the hits we got (p5).

10.The discussion is very superficial and does not provide much context for this work or address its implications.
We hope that all the new pieces in the discussion section, that were added to address the referee's points, will improve the discussion section.

Referee #3

(1) The authors have nicely shown that the ectopic expression of ARK5 causes reduction of LATS1 levels through phosphorylation of LATS1 at Ser 464. However, it is unclear how this happens. Takahashi et al (2006) reported that the reduction of WARTS/LATS1 level was attenuated by the addition of MG132, a proteasome inhibitor, in senescent cells, suggesting that LATS1 is destabilized by the proteasome dependent protein degradation machinery during cellular senescence. It is therefore important to examine whether this is also the case in ARK5 induced LATS1 reduction.

As explained above, we have added this experiment as Figure S12 and have added comments on p14. In our hands, we did not see any effect of the MG-132.

(2) Along these lines, although Takahashi et al (2006) failed to prevent reduction of LATS1 level by substitution of Ser 464 to Ala (see Supplementary information 3b), the same substitution mutant (S464A) is resistant to ARK5-induced reduction of LATS1 in this manuscript (Fig.7F). Because Takahashi et al (2006) used SVts8 cells, a conditionally immortalized human fibroblasts, and Humbert (this paper) used 293 cells, it is possible that these seemingly contradictory data are, at least partly, due to the difference of the cellular contexts in these cell lines. It is therefore important to examine whether the S464A mutant is resistant to degradation in senescent human diploid fibroblasts rather than in established cell lines. I realize this issue presents technical challenge that may be formidable, but no doubt this is very important.

In the WI-38 cells, we failed to set up the appropriate conditions to examine effect of NUAK1 over LATS1 S464. But importantly, the differences between Takahashi study and ours also include sensitivity to MG-132 treatment. It is thus possible that two different mechanisms, through different upstream kinases, resulted in LATS1 down regulation, aneuploidy and senescence. This issue is discussed on p14.

(3) Since oncogenic Ras expression is known to induce senescence like cell cycle arrest, it is interesting to know whether ARK5 expression is also induced in Ras-induced senescence. It is also interesting to know whether NUAK2 expression is increased in both replicative- and Ras-induced senescence.

We have performed qPCR analysis for NUAK1 and NUAK2 on RNA extracted from proliferating and senescing WI-38 cells and from control- and RasV12-infected WI-38 cells. These results have been added as figure S9 and comments are added in the discussion section on p13.

(4) In Fig.4D, ectopic expression of K84A significantly enhanced cellular proliferation in HDFs. Is this because K84A acts as a dominant negative against endogenous ARK5?

Indeed, these results suggested that the kinase dead mutant acts as a dominant negative form. To confirm this point, we have shown the ability of the kinase dead mutant to revert effect of NUAK1 WT to LATS1 levels. Data were presented as Figure S6 and commented p10.

(5) The quality of Fig.S3 is too poor. The authors should replace this figure with a higher quality photo.
This problem should be resolved now.

Decision letter 27 October 2009

Thank you for submitting your revised manuscript for our consideration, and sorry for the delay in getting back to you with a response. Two of the original referees had agreed to evaluate it once more, but we have - despite numerous reminders sent from our office - still not received the comments of one of them. Given that the other reviewer (original referee 1) is by and large convinced by your improvements in response to the original reviews, I will provide you with a decision now in order to avoid any further unnecessary loss of time. Thus, we shall be happy to accept your manuscript for publication in The EMBO Journal. Before we proceed with final acceptance of the study, I would however kindly ask you to modify the manuscript text according to a last request of referee 1 - that is to replace the term "genetic instability" with something like "aberrant DNA content" or "abnormal DNA content", in agreement with what you had also offered in your point-by-point response letter.

After that, we should be able to swiftly proceed with formal acceptance and production of the paper!

With best regards,

Editor
The EMBO Journal

