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p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype

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

1st Editorial Decision 22 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please excuse the slight delay in getting back to you with the results of its evaluation, but now we have finally received the comments from three expert reviewers, which are copied below. As you will see, the referees consider your new implication of p38 MAP kinase as SASP regulator interesting and potentially important for understanding the senescence-associated secretory phenotype, although referee 3 is more cautious about the overall conceptual advance conveyed by these findings in light of established links between p38 and senescence. In this situation, I conclude that we shall be able to consider your study further for publication pending adequate revision of a limited number of specific points brought up by the reviewers. Of these, the most important one reverberating in the comments of all three referees pertains to the discrepancies regarding your earlier report on SASP regulation by the DDR (Rodier et al 2009), and the relationship between SASP-DDR-p38 will therefore need to be better reconciled in one way or another in a revised manuscript.

I would thus like to invite you to prepare a revised version of the study in light of the referees comments, and should you be able to satisfactorily address the main point mentioned above as well as to the more specific technical and control issues, then we shall be happy to consider the study further for publication. I should however remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, we will also require a brief section specifying the contributions of all individual authors on the paper. 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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Work presented in this manuscript identified the p38MAPK-NF-kB pathway as an important mediator of the senescence-associated secretory phenotype, which contributes to the reinforcement of senescence and clearance of senescence cells in vivo. The findings are intriguing and are well supported by experimental data, and thus are suitable for publication in EMBO J. There are a few issues that need to be addressed.

(1) The authors concluded that activation of p38MAPK has a delayed, slow kinetics upon senescence induction, with similar kinetics to that of SASP. However, there were already significant levels of p38-P and Hsp-27-P at the earliest time point they examined (day-2) (figure 1A). It is therefore unclear when p38 and Hsp-27 first become activated. It is suggested that the analysis be extended to earlier time points, and that the rapid/transient stimuli (LPS and TNFa) be included in parallel as controls.

In addition, the kinetics of p38 activation is not consistent with the kinetics of Hsp-27 phosphorylation. Hsp-27-P peaks earlier than p38-P, despite that Hsp-27 is a downstream substrate of p38. This discrepancy needs to be discussed.

(2) It will be helpful to come up with a model to explain why both DDR and p38MAPK are both necessary and sufficient for the induction of SASP, although they are independent of each other.

Referee #2 (Remarks to the Author):

In this manuscript, Freund et al. attempt to demonstrate that p38 MAP kinase is a necessary and sufficient mediator of the senescence-associated secretory phenotype that is associated with cells induced to undergo senescence by DNA damage or oncogene overexpression. They show that diverse stimuli can activate p38 in a delayed manner (days rather than hours) and that inhibition of p38 results in a marked reduction of expression of many of the SASP factors. Moreover, constitutive activation of p38 resulted in SASP upregulation. They confirmed that p53 restrained the SASP response and that p38 regulated SASP independently of the DNA damage response pathway. The authors also demonstrated an important role in p38 upregulation of NF-kappa B signaling, with many of the NF-kB targets being SASP genes. The authors conclude that p38MAPK plays anovel and critical role in SASP regulation.

This is very well written, well referenced paper that describes an important new finding in the senescence field as it provides a mechanism for an important component of the senescence phenotype. The data is very clearly presented, augmented with key supplementary data, and the authors' conclusions are well supported by the data. This reviewer has no major criticisms.

Only a couple minor points:

(1) Figure 4D. The authors show that downregulation of ATM and Chk2 do not significantly affect IL-6 secretion 8 days after p38 activation. This seems to differ from results obtained in an earlier paper (Rodier et al., 2009) in which shATM does downregulate IL-6 secretion 9-10 days after XRA (Fig. 4b). Are these fundamentally different types of experiments, or is there a way to reconcile what

seem to be very different results?

(2) Figure 5. The authors present data showing that p38 increases NF-kB signaling and that inhibition of p38 decreases NF-kB activity and expression of SASP genes. However, it is not completely clear that increased NF-kB is directly regulating specific SASP genes (though some have NF-kB sites in their promoters). Confirmation of increased RelA binding to a couple specific SASP gene promoters after senescence or p38 activation through ChIP assays would be fairly convincing.

Referee #3 (Remarks to the Author):

Freund et al report on the identification of p38MAPK as an essential mediator of the secretory phenotype of senescent cells (SASP), acting independently of the DDR. They showed recently that the DDR is an important component of the SASP. Now they describe that independent of this, p38 is activated in senescent cells and is responsible for the induction of several SASP factors. This is mediated by NF-kB.

Previous work by the Campisi group and others has been instrumental in the identification of factors secreted by senescent cells, and in addressing the roles (both beneficial and unfavourable) of these factors in tumour progression. Recently, Campisi and coworkers found that high dose, but not low dose, radiation triggers the SASP. In this study, they link SASP to p38 and NF-kB, providing some new insight in the mechanism by which the SASP is regulated. The data are generally of good quality and most claims are sufficiently supported by the results.

However, the paper merely connects elements that all have previously been well established, by numerous groups. Although the paper provides some new insight, it does not really add a new element that brings the field a significant step further. Most importantly, a dominant role for p38 and its regulators (like MKK6) in cellular senescence (and its regulators like p16), be it induced by oncogenes like Ras or telomere shortening, and in tumour progression has been demonstrated previously (to name a few: Bulavin 2002/3/4, Wang 2002, Wong 2009, Zdanov 2006, Hong 2010). As several papers have shown that the SASP is a common response to senescence inducing signals, the current data extend our understanding, but to a relatively limited extent.

A similar comment should be made for the link between p38 and NF-kB (see review Karin 2005), between NF-kB and senescence (e.g. Acosta 2008) and for the role of p38 in cancer cell migration (e.g. Kumar 2010) and the DDR (review Han 2007) - also these elements have been reported by several others previously. Connecting this to the SASP is new but again, the SASP is known to commonly accompany senescence so the step forward is small.

Furthermore, as is outlined by the authors (p. 8, bottom), the DDR accounts only for a portion of the SASP response that is induced by oncogenic Ras. This seems to contrast somewhat with their recent report (Rodier 2009) emphasising the role of the DDR in SASP. But it also raises the question how important the DDR really is for the senescence response. Indeed, in contrast to their previous paper, here the authors make a case that p38 regulates the SASP independent of DNA damage. Although this particular finding is unexpected and therefore of interest, it leaves room for confusion in light of what the authors have published previously (see also comment below).

Finally, no attempt was made to study whether or how p38-SASP signalling is perturbed in tumour cells, in vitro or in vivo.

Other comments:
-Figure 4c-e would be significantly strengthened by additional controls: in c, all samples shown in b should be included for reference, in d and e as well as their supplementary figures Xray and Ras should be included.
-p.4: "However, the DDR is not sufficient for the SASP." - This is confusing as this group reported in the Rodier et al paper that the SASP develops when the damage is sufficient to generate PDDF and persistent DDR signalling, arguing that the DDR, if persistent, does suffice to induce the SASP.

1st Revision - authors' response 16 February 2011

Thank you for handling our manuscript and giving us an opportunity to respond to the referees' comments, which we found very useful. Below, please find our point-by-point response to the referees. As you will see, we have answered all the substantive comments of the referees by new data now included in the revised manuscript, or clarifying some of the points we failed to make clearly.

Referee #1 (Remarks to the Author):

Work presented in this manuscript identified the p38MAPK-NF-kB pathway as an important mediator of the senescence-associated secretory phenotype, which contributes to the reinforcement of senescence and clearance of senescence cells in vivo. The findings are intriguing and are well supported by experimental data, and thus are suitable for publication in EMBO J. There are a few issues that need to be addressed.

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This is a valid comment. In the revised manuscript, we now show that, while LPS stimulation induces rapid (within 1 hour) and transient phosphorylation of p38MAPK within the first 24 hours after addition, XRA induces only a negligible increase in p38MAPK phosphorylation within the first 24 hours (new Figure S1C). The majority of p38MAPK phosphorylation occurs at later timepoints (>2 days after XRA), as shown in our original manuscript.

In addition, the kinetics of p38 activation is not consistent with the kinetics of Hsp-27 phosphorylation. Hsp-27-P peaks earlier than p38-P, despite that Hsp-27 is a downstream substrate of p38. This discrepancy needs to be discussed.

We agree with the referee that the kinetics of Hsp27 phosphorylation are not identical to the kinetics of p38 phosphorylation, with Hsp27 phosphorylation peaking earlier. We have verified this in multiple independent experiments. However, we do not believe these data contain a discrepancy. The steady state phosphorylation level of any downstream target of a MAP kinase is modulated by multiple factors, including rate of protein turnover, phosphatase activity, and the enzyme kinetics of the upstream kinase. Our data suggest that the level of active p38MAPK reached in SEN(XRA) cells is only sufficient to achieve a certain steady-state level of phosphorylated Hsp27, which is reached by day 8. This may be to due the enzyme kinetics of p38MAPK itself (i.e. a threshold effect), or any number of other cellular processes. Notably, higher levels of p38MAPK activity, such as those achieved by MKK6EE, do push Hsp27 phosphorylation past this plateau (new Figure S4F), suggesting these data are not a result of film saturation or other experimental artifact.

(2) It will be helpful to come up with a model to explain why both DDR and p38MAPK are both necessary and sufficient for the induction of SASP, although they are independent of each other.

This is an important point raised in slightly different ways by all three referees. We recognize that our initial explanation of the relationship between DDR-SASP regulation (Rodier et al., 2009) and p38MAPK-SASP regulation was unclear. We have updated the manuscript text and the figures in multiple ways to address this issue:

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First, we would like to clarify the distinction between any DNA damage response, which of course includes every cellular process that changes after DNA damage (XRA), and the canonical DDR, which we studied in our 2009 Nat Cell Bio paper (Rodier et al., 2009). p38MAPK activation is certainly a response to DNA damage, but it falls outside the standard definition of the canonical DDR, which is limited to factors directly involved in the sensing and repair of specific sites of DNA damage. Canonical DDR proteins include ATM, ATR, DNA-PK, CHK1, CHK2, etc. Using this definition of the DDR, we conclude that, though canonical DDR activity is necessary for the SASP (Rodier et al., 2009), its signaling is not sufficient, as it reaches peak activity within hours after XRA ñ many days before the SASP is activated. Therefore, there must be a slower, cooperating event, which we show is p38MAPK. We show that p38MAPK activity is both necessary for the SASP after XRA or RAS and is sufficient for the SASP when maximally activated (i.e. by MKK6EE). To clarify this point in the text, we have included several additional sentences in the introduction to clarify that we are contrasting the canonical DDR pathway to the p38MAPK pathway, and we highlight which DDR factors are included in the ìcanonicalî category.

Nevertheless, as the referees point out, an important question remains: if p38MAPK is sufficient for the SASP, but independent of the DDR, how can the DDR be necessary for the SASP? We have developed a model that is supported by additional data that we have added to the manuscript. We now show that p38MAPK activation is sufficient for the SASP only when its signaling reaches a high level, above the level induced by XRA or RAS (new Figures S4F, S4G). We show that MKK6EE expression induces this high level of endogenous p38MAPK phosphorylation and is sufficient to induce full SASP activity. However, when we blunted p38MAPK activity in MKK6EE-expressing cells by treating with varying doses of SB203580, we found that p38MAPK activity at the level induced by XRA (as determined by Hsp27-P) was insufficient to induce an increase in IL-6. Consequently, we conclude that the level of p38MAPK signaling in SEN(XRA) cells is not sufficient to induce the SASP, though at higher levels it is.

Thus, our present manuscript and Nat Cell Biol paper are consistent, and the seeming discrepancy is the result of a signaling threshold.

In the revised manuscript, we suggest a working model, the detailed testing of which is beyond the scope of this manuscript, to explain our results. We propose that the threshold effect could be caused by p38MAPK (or one of its downstream targets) having varying degrees of efficiencies in phosphorylating multiple phosphorylation sites on RelA. RelA is known to require phosphorylation on multiple sites for maximal DNA binding and transcriptional activity. We hypothesize that p38MAPK has a high affinity for certain RelA sites, which are phosphorylated by the amount of p38MAPK-P caused by XRA, but low affinity for others, which are phosphorylated only by high levels of p38MAPK-P. In SEN(XRA) and SEN(RAS) cells, then, those low affinity sites are not phosphorylated by p38MAPK, but rather by components of the canonical DDR, making both p38MAPK and the DDR necessary for the SASP. This model makes several predictions, two of which we tested in the revised manuscript: 1) knockdown of DDR factors should decrease NF-kB transcriptional activity in SEN(XRA) cells, and 2) the residual NF-kB transcriptional activity seen in SEN(XRA)+SB cells should be decreased by DDR knockdown. We found that both these predictions hold (new Figure 5E). We have added a description of this working model to the Discussion.

Referee #2 (Remarks to the Author):

In this manuscript, Freund et al. attempt to demonstrate that p38 MAP kinase is a necessary and sufficient mediator of the senescence-associated secretory phenotype that is associated with cells induced to undergo senescence by DNA damage or oncogene overexpression. They show that diverse stimuli can activate p38 in a delayed manner (days rather than hours) and that inhibition of p38 results in a marked reduction of expression of many of the SASP factors. Moreover, constitutive activation of p38 resulted in SASP upregulation. They confirmed that p53 restrained the SASP response and that p38 regulated SASP independently of the DNA damage response pathway. The authors also demonstrated an important role in p38 upregulation of NF-kappa B signaling, with many of the NF-kB targets being SASP genes. The authors conclude that p38MAPK plays anovel and critical role in SASP regulation.

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In this manuscript, we showed that ATM and CHK2 knockdown have no effect on the MKK6EE-induced SASP, whereas our previous paper shows that ATM and CHK2 knockdown decreases the XRA-induced SASP. We recognize that this point was unclear in the original version and have included the SEN(XRA) and SEN(RAS) controls when examining the ATM and CHK2 knockdowns in MKK6EE cells to highlight the difference between the experiments (new Figure 4D). For a more detailed explanation of why this difference between SEN(XRA)/SEN(RAS) and MKK6EE exists, please see our response to referee #1, who raised a similar point (point #2).

(2) Figure 5. The authors present data showing that p38 increases NF-kB signaling and that inhibition of p38 decreases NF-kB activity and expression of SASP genes. However, it is not completely clear that increased NF-kB is directly regulating specific SASP genes (though some have NF-kB sites in their promoters). Confirmation of increased RelA binding to a couple specific SASP gene promoters after senescence or p38 activation through ChIP assays would be fairly convincing. We agree with the referee that this is a useful additional experiment. We now provide chromatin immunoprecipitation data showing increased RelA binding to the IL-6, IL-8, and GM-CSF promoters in SEN(XRA) cells (new Figure 5D).

Referee #3 (Remarks to the Author):

Freund et al report on the identification of p38MAPK as an essential mediator of the secretory phenotype of senescent cells (SASP), acting independently of the DDR. They showed recently that the DDR is an important component of the SASP. Now they describe that independent of this, p38 is activated in senescent cells and is responsible for the induction of several SASP factors. This is mediated by NF-kB.

Previous work by the Campisi group and others has been instrumental in the identification of factors secreted by senescent cells, and in addressing the roles (both beneficial and unfavourable) of these factors in tumour progression. Recently, Campisi and coworkers found that high dose, but not low dose, radiation triggers the SASP. In this study, they link SASP to p38 and NF-kB, providing some new insight in the mechanism by which the SASP is regulated. The data are generally of good quality and most claims are sufficiently supported by the results.

However, the paper merely connects elements that all have previously been well established, by numerous groups. Although the paper provides some new insight, it does not really add a new element that brings the field a significant step further. Most importantly, a dominant role for p38 and its regulators (like MKK6) in cellular senescence (and its regulators like p16), be it induced by oncogenes like Ras or telomere shortening, and in tumour progression has been demonstrated previously (to name a few: Bulavin 2002/3/4, Wang 2002, Wong 2009, Zdanov 2006, Hong 2010). As several papers have shown that the SASP is a common response to senescence inducing signals, the current data extend our understanding, but to a relatively limited extent.

Our previous work demonstrated that the SASP and growth arrest are regulated in quite different ways. Though they are both part of the senescence program and are often induced by the same upstream stimulus, the conclusion that a positive regulator of the senescence growth arrest must also be a positive regulator of the SASP is not obvious, and is, in fact, not true for the two most common growth arrest regulators: p16 and p53. Our prior work has shown that neither p53 nor p16 are

positive SASP regulators, despite being potent positive senescence growth arrest regulators.

We hold that understanding the connection between the SASP and growth arrest is quite important, as p38MAPK represents a regulator of both the SASP (this paper) and the growth arrest (the papers cited by the referee). As p38MAPK downstream targets, such as PRAK and p16, do not seem to regulate the inflammatory cytokine response to senescence-inducing stimuli, the evidence in our manuscript suggests that p38 may serve as one of the most downstream common nodes between the two arms of senescence (growth arrest and SASP).

A similar comment should be made for the link between p38 and NF-kB (see review Karin 2005), between NF-kB and senescence (e.g. Acosta 2008) and for the role of p38 in cancer cell migration (e.g. Kumar 2010) and the DDR (review Han 2007) - also these elements have been reported by several others previously. Connecting this to the SASP is new but again, the SASP is known to commonly accompany senescence so the step forward is small.

In this manuscript we demonstrate that the activation of p38MAPK and NF-kB at senescence is slow and chronic ñ a markedly different response from the rapid, transient, self-quenching response that occurs in response to most other stimuli. We have also shown that p38MAPK and the DDR are independently regulated, which again is different from the crosstalk observed in studies of acute stresses. Thus, our data suggest that the activation and cooperation of these pathways at senescence represents a novel form of regulation - one that has not addressed by previous studies. Though we appreciate the referee's critique, several of the reviews that the referee cites are only superficially related to our report. For example, the role of p38MAPK in cancer cell migration that the referee cites is a cell-autonomous effect in which tumor cells activate p38MAPK to promote their own invasion ñ a process that is different both in mechanism and in evolutionary selection than the paracrine effect that we describe here.

Furthermore, as is outlined by the authors (p. 8, bottom), the DDR accounts only for a portion of the SASP response that is induced by oncogenic Ras. This seems to contrast somewhat with their recent report (Rodier 2009) emphasising the role of the DDR in SASP. But it also raises the question how important the DDR really is for the senescence response. Indeed, in contrast to their previous paper, here the authors make a case that p38 regulates the SASP independent of DNA damage. Although this particular finding is unexpected and therefore of interest, it leaves room for confusion in light of what the authors have published previously (see also comment below).

We are not clear which passage the referee is referring to ñ prior to this revision, we included no data showing the role of the DDR in the regulation of the SEN(RAS) SASP, and any discussion of the role of the DDR in SASP regulation was based on our previous report (Rodier 2009). However, in the revised version of our manuscript, we provide new data (new Figure 4D) demonstrating the role of ATM in the regulation of IL-6, IL-8, and GM-CSF; these data are in agreement with our previous report. In this manuscript, we make the point that p38MAPK acts independently of the canonical DDR, cooperates with the DDR to regulate the SEN(XRA) and SEN(RAS) SASPs, and can activate the SASP without the DDR when activated at a high level (notably, the converse is not true ñ the DDR cannot activate the SASP without p38MAPK, as is evidenced by the lack of SASP activity in the 24 hours immediately after XRA, when DDR activity is maximal but p38 is not activated). For a more detailed discussion of the cooperation between the DDR and p38 pathways, please see our response to referee #1, point #2.

Finally, no attempt was made to study whether or how p38-SASP signalling is perturbed in tumour cells, in vitro or in vivo.

We agree with the referee that such studies would add to the interest of the manuscript. We studied two tumor cell lines ñ MCF-7 and MDA-MB-231. We found that basal levels of phosphorylated p38MAPK were normal in MCF-7 cells and very high in MDA-MB-231 cells. Interestingly, XRA actually decreased p38MAPK phosphorylation in both cell lines 8 d later, and this correlated with a lack of IL-6 induction. One interpretation is that these tumor cell lines inactivate the p38MAPK pathway to prevent the SASP and p16 induction and to allow their continued proliferation. However, like many tumor cell lines, both MCF-7 and MDA-MB-231 populations underwent widespread (~50%) apoptosis after irradiation, meaning that the decrease in p38MAPK phosphorylation may alternatively be an artifact of survival selection ñ it is known that activation of

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p38MAPK induces apoptosis in some cell types, particularly tumor cell lines. Therefore, we think that any definitive conclusions would require extensive further study in a wide variety of tumor cells. While such experiments would be interesting, we suggest that it is important to describe the regulation of the SASP and p38MAPK pathway in normal cells during senescence before moving on to the deregulation of this pathway in a background as complex and heterogeneous as cancer.

Other comments:
-Figure 4c-e would be significantly strengthened by additional controls: in c, all samples shown in b should be included for reference, in d and e as well as their supplementary figures Xray and Ras should be included.

The referee makes a valid point that additional controls would be informative ñ we have included XRA and RAS data in Figure 4D, and added RAS data to Figure 4E, which already included XRA data.

Adding senescent XRA and RAS controls to Figure 4C would not be particularly instructive, as global levels of phosphorylated DDR proteins such as p53, ATM and CHK2 would be indistinguishable from PRE controls: cells induced to senesce by XRA or RAS show a global increase in activated DDR factors only in the early stages of senescence (see Figure 4A). After global levels decline, the relatively small amount of p53-P, ATM-P, and CHK2-P remaining is confined to the persistent DNA damage foci (PDDF) mentioned by the referee, and it is these foci that are responsible for senescence regulation, as demonstrated in our previous paper (Rodier et al., 2009). However, we did examine PDDF (using 53BP1 as a marker) in Figure 4B and saw no increase in senescent MKK6EE cells.

Because cells that are fully senescent due to XRA or RAS do not have a global increase in p53-P, ATM-P, or CHK2-P, we did not expect there to be a global increase in these proteins in senescent MKK6EE cells. We originally included the Figure 4C western to simply confirm our expectation. However, we recognize that this late-stage western does not preclude an early increase in global DDR activation. In order to further demonstrate that there is no increase in DDR signaling during early timepoints after MKK6EE expression, we add in the revised manuscript a time course examining p53 phosphorylation on Ser15 after RAS and MKK6EE infection (new Figure S4D). RAS induced p53-P early during the hyperproliferative phase, as expected, which then declined. However, at no point did MKK6EE induce any detectable p53-P.

Lastly, the XRA and RAS controls for Figure S4E are in Figure 4E ñ they are not combined into a single figure because doing so would interrupt the order in which these concepts are introduced in the text.

We hope these changes address the reviewer's concerns.

-p.4: "However, the DDR is not sufficient for the SASP." - This is confusing as this group reported in the Rodier et al paper that the SASP develops when the damage is sufficient to generate PDDF and persistent DDR signalling, arguing that the DDR, if persistent, does suffice to induce the SASP.

The referee makes the valid point that all downstream effects of direct DNA damage (i.e. XRA) are technically a DNA damage response, and thus the set of all cellular effects that exist several days after XRA are a persistent DDR and are sufficient for the SASP. However, in order to study the different pathways that are activated as part of senescence, we are using ìDDRî in its canonical form, which is limited to those factors involved in the rapid sensing and repairing of sites of DNA damage (e.g. ATM, ATR, DNA-PK, CHK1, CHK2, etc). We recognize that this distinction was unclear in the original draft and have updated the Introduction to clarify the terminology. Referee #1 raised a similar point. Please see our response to referee #1, point #2 for further discussion.

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Thank you for submitting your 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.

Thank you again for having chosen The EMBO Journal for publication of this interesting story!

Yours sincerely,

Editor
The EMBO Journal

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