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Aging and DNA-damage response activate a regulatory pathway involving miR-29 and p53

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1st Editorial Decision 12 August 2010

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, all three reviewers find your results linking miRNAs, aging and DNA damage interesting and potentially important. They nevertheless also raise a number of concerns that would need to be addressed prior to publication. Given that most of these points refer to specific (rather than conceptual) issues, I would like to give you the opportunity to answer to them in the form of a revised version of the manuscript. Should you be able to satisfactorily address the main criticisms, then we shall be happy to consider a revised manuscript further for publication. One key point for the eventual success of such a revision will however be the addition of data to strengthen the physiological relevance of your findings with regard to aging/cellular senescence/cancer, as mirrored in the comments of all three reviewers.

Please keep in mind 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 various 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, please make sure to carefully assemble and proofread the final version also from an editorial point of view, paying attention to our format guidelines e.g. for the order of manuscript sections, formatting of references in the bibliography, completeness of in-text citations, and also briefly indicate the individual author's contributions, either in the acknowledgements section or in an adjacent separate section. 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):

The manuscript entitled "Aging and DNA-damage response activate a regulatory pathway involving miR-29 and p53" by Ugalde AP et al., addresses the impact of miRNAs in DNA damage-driven Zmpste-24 progeria as well as in natural aging. Previously, the Lopez-Otin group has done much and important work to unravel the pathological mechanisms leading to the accelerating aging in Zmpste 24-/- mice. Their current effort to address the role of (selected) miRNAs in accelerated aging and DNA damage is commendable, novel and with high relevance to the aging field. That being said, however, several points require further clarification and/or experimentation that, if properly addressed, they will greatly improve the content of the work presented in this manuscript.

1. The authors conducted an miRNA expression analysis in Zmpste-24-/- and wt mice (figure 1A). This approach was aimed at identifying potential miRNAs implicated in Zmpste-24 progeria and likely also in natural aging. Subsequently, the authors have taken a forward approach to identify miR-29 sequence elements in selected gene targets. I am aware that in a previous work (Varela et al., 2005), the Lopez-Otin group analyzed the complete mouse transcriptome in Zmpste 24-/- mice. Having used a complete mouse gene expression platform (Mouse Affymetrix 430A), it would have been particularly encouraging if the authors attempt to correlate the present miRNA and previous gene expression profiles from Zmpste-24 mice. By analogy, does any of the identified genes in Figure 3 are also seen in their previous gene expression profiling studies (at least at the mRNA level)? In view of the previous results such an unbiased comparison would have revealed the potential impact of miR29 family of miRNAs and others in regulating gene expression at the RNA level in Zmpste-24-/- mice. A similar approach could also be considered using natural aging gene expression profiles (several Affymetrix datasets are available for liver and muscle that make compatible their comparative analysis to the Zmpste-24-/- datasets as well as the miRNAs profiles).

2. In e.g. figure 1B (and elsewhere), it is important to provide information on the number of animals tested. Do the standard deviations (or else standard error of the means) of each column bar shown reflect technical (e.g. same PCR reaction performed e.g. three times) or biological replicates (e.g. 3 animals per group examined)?

3. In page 10, line 5 the authors state that "Finally, we examined the putative correlation....the highest levels of miR-29 miRNAs". The authors need to clearly define which progeroid features they considered in their analysis, how did they measure "exacerbation" and how the animal selection and correlation was performed. For instance, if shorter lifespan is among those "exacerbated" progeroid features (shorter than one would expect for Zmpste 24-/- mice) did the authors also account for age-dependent differences? Where the examined animals moribund?

4. Figure 2 is aimed at describing the relevance of miR-29 to DNA damage. To this end, the relevance of replicative senescence (shown in figure 2A) to the general scope of figure 2 (the impact of DNA damage) is somewhat questionable. If the authors assume that DNA damage increases with increasing cell passage then they will need to show it (along with senescence). As doxorubicin induces single and double strand breaks, the authors could provide evidence for e.g. increased gH2aX foci (for DSBs) and b-gal staining (for senescence) with increasing cell passage. These endpoints are not a priori determined in the study and only a vague assumption is being made.

5. The authors refer to the down-regulation of miR-29 in Ames dwarf mice. This is an interesting

observation that could be potentially examined in the context of this work as well. For instance, it makes one wonder whether down-regulation of miR-29 could potentially delay replicative senescence in the tested cell cultures employed in this study. Such experiments, however, can only be carried out once proper markers (gH2aX, b-galactosidase or other) have been thoroughly established in the aforementioned system.

6. Besides single and double strand breaks, Doxorubicin induces a potent G2/M arrest. How the authors exclude that the miR29 upregulation (compared to untreated wt cells) is not due to cell cycle differences (rather than DNA damage)? Were the untreated cells carried along with the treated ones (time vehicle control) during cell culturing or were the control cells taken at "zero" timepoint (before treatment)? This point should be clarified.

7. Doxorubicin is used throughout the work as the sole DNA damage-inducing treatment. To strengthen the relevance of DNA damage to miR-29 upregulation the authors should try additional genotoxic agents (e.g. UV, mitomycin, 4NQO, H202). For instance, doxorubicin induces permanent lesions or lesions that are difficult to repair whereas H202 only induces transient ones that are rapidly and efficiently repaired. Would miR-29 upregulation be seen upon exposure of cells to both treatments? This is important to judge whether the up-regulation of miR-29 is relevant to lesions accumulating gradually upon natural aging or else rapidly upon progeria.

8. The authors show evidence that miR-29 is upregulated upon DNA damage and that the miR-29 family of miRNAs is implicated in the response to DNA damage. If these important claims are to be made, then the authors should also provide evidence that cells impaired in miR-29 or else treated with anti-miR29 are more sensitive to doxorubicin or g-irradiation (both induce double strand breaks). In addition, the authors could test the expression levels of miR-29 in cell lines defective in ATM (deals mainly with the response to DSBs) as opposed to ATR (deals mainly with the response to UV-induced transcription-blocking DNA lesions). Without such in vitro functional evidence, the conclusion(s) drawn in the manuscript are rather weakly justified.

9. Along the same lines, the physiological in vivo relevance of miR-29 to DNA damage is lacking. For instance the authors could test the expression of miR-29 in progeroid, DNA repair-deficient mice (e.g. NER or other). The advantage of examining the expression of miR-29 in tissues derived from DNA repair-deficient mice is that the relevance of miR-29 to DNA damage will then be examined upon intrinsic rather than exogenous genotoxic sources, thereby making it more relevant to the natural aging situation.

10. On the proposed model (page 17, last paragraph): The authors should take into consideration that Ames dwarf mice that show down-regulation of miR-29 are long-lived animals primarily due to the fact that they show very low tumor frequency. Isn't that result (i.e. the down-regulation of miR-29 in a tumor-free model) somehow contradictory to what the authors proposed in page 17, last paragraph (the potential role of miR29 in tumor predisposition)? If possible an additional explanation should be discussed.

11. Indeed miR-29 appears to increase upon aging but the authors have not adequately clarified why. Do they assume it is due to gradual accumulation of DNA damage with advancing age? Perhaps a model figure could be generated that will discuss their proposed hypothesis in a rather more informative context (highlighting the primary instigator of the response and the relevant endpoints).

12. The chosen title is somewhat puzzling: Is it the DNA-damage response that activates a regulatory pathway involving miR-29 or the DNA damage itself?

Referee #2 (Remarks to the Author):

In this manuscript, Ugalde et al. start by analyzing miRNA species expression in wildtype and Zmpste24-deficient mice, which exhibit premature aging phenotpes. They have identified miR-29a, miR-29b, and miR-29c as a family of microRNAs that are consistently upregulated in liver and muscle of the Zmpste24-deficient mice and in old wildtype mice. They go on to show that miR-29s are upregulated in DNA damaged and late passaged MEFs in a p53-dependent manner. Using a number of bioinformatics tools they identify a number of potential miR-29 targets and confirm that

these genes are indeed targeted in a luciferase-based assay. Particular attention as a mR-29 target is paid to Wip1, a phosphatase that regulates p53 and ATM signaling. The authors show in human cancer cell lines that miR-29 species do suppress Wip1 protein levels and presumably function as measured by phospho-p53 levels. The authors propose the existence of a novel regulatory circuitry involving miR-29, Ppm1d and p53, which is activated in aging and in response to DNA damage.

This is a well written, very exciting paper which reveals a potential new microRNA-based aging/DNA damage-associated pathway and as such would be of general interest to EMBO Journal readers. While the data here is fairly convincing regarding this miR-29 pathway, the story could be made even more compelling if a few additional experiments could be performed.

First, the data showing that p53 regulates miR-29s is confined to Fig. 2C and is limited in nature. How does p53 upregulate miR-29s? Is it through direct transcriptional upregulation? Did the authors look for p53 RE consensus sites in the miR-29 promoter? What about miR-29 promoter-luciferase experiments with and without p53? Or a ChIP assay with p53 Ab on the miR-29 promoter?

Second, the finding that miR-29s suppress Wip1 protein levels in cancer cell lines is exciting. But what about in wildtype vs. Zmpste24-/- MEFs or in wildtype vs. Zmpste24-/- tissues? Such experiments would more directly show the relevance of this pathway in the premature aging phenotypes exhibited by the Zmpste24-/- mice.

Other points:

(1) Figure 2A: Only miR-29b is shown. Were the results for miR-29a and miR-29c similar?

(2) Figure 5A, 5B: The gene being assayed (narf or PPM1D) should be indicated in the Figure.

(3) Figure 6A: Wip1 levels in the untreated MCF-7 cells seem almost non-existent. Even unstressed MCF-7 cells normally have very high levels of Wip1. Are the authors confident about the quality of their Wip1 antibodies? Or is this just a short film exposure?

(4) Figure 6C,D: Measuring Wip1 functional effects only by assaying phospho-p53 levels seems somewhat inadequate. The authors should at least include levels of p53 protein as well as phospho-p53 to see the relative levels of p53 stabilization as well as prove that phospho-p53 differences are due specifically to differences in phosphorylation and not p53 protein levels. Also, showing effects on phosphorylation levels of one or more other known Wip1 targets would be particularly compelling. Finally, Wip1 protein levels should also be shown in these panels to complete the story.

(5) Figure 5,6: Controls used for miR-29s were usually empty vectors or mutant miRs. Were antagomirs to miR-29s used to actually decrease miR-29 expression. Showing increased Wip1 functional activity with antagomirs would be particularly impressive.

Referee #3 (Remarks to the Author):

In this manuscript Ugalde et al. report upregulation of the miR-29 family of miRNAs in Zmpste24 -/- mice, a model of accelerated aging, and in physiologically aged mice. Upregulation of miR-29 miRNAs is associated with cellular senescence in vitro and is induced by the DNA damage agent Doxorubicin in a p53-dependent way. The authors identify the protein phosphatase Pmp1d as a target of the miR-29 miRNAs and show that increased levels of phosphorylated p53 are associated with ectopic expression of miR-29 miRNAs in U2OS cells. Based on these observations and their previous findings showing p53 signaling activation in Zmpste24 -/- mice, the authors propose that miR-29 miRNAs have a functional role in mediating the DNA damage response and the senescent phenotype in accelerated and physiological aging.

The study addresses an interesting question. miRNAs are being implicated in a growing number of cellular and physiological processes and very little is known about their role in the aging process. Zmpste24 -/- mice show a dramatic accelerating aging phenotype and represent a good model system to uncover mechanisms of aging. The upregulation of miR-29 in Zmpste24 -/- mice is clear, in particular in muscle, and the similarity with normally aged mice is remarkable. The fact that

Ppm1d is a novel miR-29 target and that its upregulation upon DNA damage is inhibited by the microRNAs is also convincingly shown. However, these observations are circumstantial with respect to aging, and the functional link between miR-29, DNA damage, p53, Pmp1d, and aging is not clear. Most of the experiments are performed using artificial systems, human cell lines instead of Zmpste -/- cells, in which acute DNA damage is transiently induced and their physiological relevance is questionable. The study would be greatly improved by showing a functional effect of miR-29/ Pmp1d on the p53-mediated cellular senescence which characterizes Zmpste24 -/- mice. See below for more specific points. If such an effect cannot be convincingly shown all the conclusions about aging should be toned down and the manuscript should only focus on the link between miR-29/ Pmp1d and DNA damage.

Major points:
1) A functional link needs to be shown between miR-29 and aging (admittedly difficult) or at least cellular senesce. The authors have previously shown that Zmpste -/- MEFs show accelerated senescence (Varela et at. 2005) presumably due to high levels of DNA damage. What is the expression level of miR-29 miRNAs in Zmpste -/- MEFs over passages compared to wt MEFs (Fig.2a)? Also, and more importantly, can anti-miR-29 rescue the accelerated senescence of Zmpste -/- MEFs? The authors show many experiments involving ectopic expression of miR-29 but a convincing experiment showing a cellular effect of anti-miR-29 is needed to demonstrate a functional role of miR-29 upregulation in aging.

2) The model proposed by the author is that miR-29 miRNAs inhibit the phosphatase Pmp1d, thereby increasing p53-posphorylation and inducing a chronic DNA damage response. This, again, must be proven in a more relevant system than U2OS. The experiments shown in fig. 6C and 6D need be performed using wt and Zmpste24-/- MEFs and correlated with cellular senescence. Ectopic expression of miR-29 in wt cells is expected to increase p53-phosporylation and induce senescence while anti-miR-29 in Zmpste24-/- MEFs should have opposite effects.

3) Fig. 6C. The total levels of p53 need to be shown to demonstrate that miR-29 act indeed through Pmp1d and not by simply stabilizing p53 and increasing the protein levels as previously shown. Furthermore, the interpretation that miR-29 expressing cells show an exacerbated response to DNA damage and a delayed one to caffeine is questionable. The increase in p53 phosphorylation needs to be normalized to the basal levels (no doxorubicin), which are higher in miR-29 expressing cells. Upon normalization the response to DNA damage and caffeine is identical or even lower than the control.

Minor points:
1) The result showing that upregulation of miR-29 upon induction of DNA damage is abolished in p53 -/- cells is interesting. Do p53 -/- cells also fail to upregulate miR-29 over passages?

2) It is not clear why the authors use MCF7 cells to establish that Pmp1d is a target of miR-29 and then switch to U2OS cells for further validation of their hypothesis. In general, too many different cell lines are used in this study (MEFs, HEK293, MCF7 and U2OS) and this makes it hard to compare the different results.

3) Fig. 6B. The claimed increase in Pmp1d levels in anti-mir expressing cells is not clear at all. A 1.2-fold increase detected by western blot (without standard deviation) cannot be trusted.

4) The final model proposed by the authors is not clear. On one hand they seem to suggest that downregulation of Pmp1d by miR-29 leads to increased phosphorylation of p53 and this contributes to the activation a chronic, abnormally high, response to DNA damage and consequent senescence. On the other hand, in the discussion at p16 the authors speculate that reduced levels of Pmp1d sensitize Zmpste24 -/- cells to genotoxic stress, somehow independently of p53, and this in turn leads to activation of p53 signaling pathway. Please explain better.

1st Revision - authors' response 18 December 2010

Please find enclosed a revised version of our manuscript now entitled "Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53" (EMBOJ-2010-75490) by Ugalde et al. We acknowledge the positive comments raised by the experts who have revised our paper as well as the opportunity to submit a revised version of our work. We have performed a number of new experiments and extensively revised our manuscript in response to the criticisms raised by the reviewers. Their comments and criticisms have been dealt with as follows:

Referee 1

1. The authors conducted an miRNA expression analysis in Zmpste-24-/- and wt mice (figure 1A). This approach was aimed at identifying potential miRNAs implicated in Zmpste-24 progeria and likely also in natural aging. Subsequently, the authors have taken a forward approach to identify miR-29 sequence elements in selected gene targets. I am aware that in a previous work (Varela et al., 2005), the Lopez-Otin group analyzed the complete mouse transcriptome in Zmpste 24-/- mice. Having used a complete mouse gene expression platform (Mouse Affymetrix 430A), it would have been particularly encouraging if the authors attempt to correlate the present miRNA and previous gene expression profiles from Zmpste-24 mice. By analogy, does any of the identified genes in Figure 3 are also seen in their previous gene expression profiling studies (at least at the mRNA level)? In view of the previous results such an unbiased comparison would have revealed the potential impact of miR29 family of miRNAs and others in regulating gene expression at the RNA level in Zmpste-24-/- mice. A similar approach could also be considered using natural aging gene expression profiles (several Affymetrix datasets are available for liver and muscle that make compatible their comparative analysis to the Zmpste-24-/- datasets as well as the miRNAs profiles).

This is an excellent suggestion and we have incorporated a comparative overview of our previously published mouse transcriptome in Zmpste24-/- mice with data on expression of miR-29 targets. We have also tried to perform a similar comparative analysis with several natural aging gene expression profiles but the enormous variability in the available data from the literature has made virtually impossible to get any clear conclusion in this regard. In relation to the analysis of Zmpste24-/- transcriptome, we have focused our analysis on samples from skeletal muscle as this was the tissue in which the observed differences in miR-29 expression levels were more evident. Importantly, in addition to demonstrating significant overlap in the analyzed datasets, and in accordance with your suggestion, we have identified those genes that are targets for miR29 (see Supplementary Fig. 1B in the revised manuscript). We want also to mention that the gene expression platform used in our previous work (Varela et al., Nature 2005) lacked probes for a number of genes which are now present in the most widely used mouse gene expression platforms. This is the case of genes important in the context of the present work (Ppm1d, Hbp1 and Narf), whose expression in tissues from Zmpste24-null mice has been analyzed by RT-PCR. These experiments (shown in Suppl. Fig. 1C of the revised manuscript) have revealed a clear down-regulation of Narf expression levels and no significant changes in Hbp1 and Ppm1d transcriptional levels, although a trend towards down-regulation in the mutant tissues was observed for all transcripts.

2. In e.g. figure 1B (and elsewhere), it is important to provide information on the number of animals tested. Do the standard deviations (or else standard error of the means) of each column bar shown reflect technical (e.g. same PCR reaction performed e.g. three times) or biological replicates (e.g. 3 animals per group examined)?

In all cases the error bars represent biological replicates. Following the reviewerís suggestion and to improve the clarity of the manuscript, we have included in the figure legends detailed information about the number and nature of the replicates.

3. In page 10, line 5 the authors state that "Finally, we examined the putative correlation....the highest levels of miR-29 miRNAs". The authors need to clearly define which progeroid features they considered in their analysis, how did they measure "exacerbation" and how the animal selection and correlation was performed. For instance, if shorter lifespan is among those "exacerbated" progeroid features (shorter than one would expect for Zmpste 24-/- mice) did the authors also account for age-dependent differences? Where the examined animals moribund?

To make the correlation between miR-29 levels and the development of the progeroid phenotype, we created two groups of Zmpste24-/- mice. The low-phenotype group was composed of 2-month-old mutant mice, which were largely indistinguishable from their wild-type littermates. The high-phenotype group consisted of 4-month-old mutant mice which, while not moribund, demonstrated severe phenotypic features due to accelerated aging, such as extensive hair and weight loss, as well as prominent kyphosis. For clarity, in the revised manuscript we have rewritten the section identified by the reviewer on page 10.

4. Figure 2 is aimed at describing the relevance of miR-29 to DNA damage. To this end, the relevance of replicative senescence (shown in figure 2A) to the general scope of figure 2 (the impact of DNA damage) is somewhat questionable. If the authors assume that DNA damage increases with increasing cell passage then they will need to show it (along with senescence). As doxorubicin induces single and double strand breaks, the authors could provide evidence for e.g. increased gH2aX foci (for DSBs) and b-gal staining (for senescence) with increasing cell passage. These endpoints are not a priori determined in the study and only a vague assumption is being made.

We agree that both senescence and DNA damage must be assessed in the cell populations in which the miR-29 family is being quantified. Accordingly, we have repeated this series of experiments using wild-type and Zmpste24-/- primary fibroblasts that had undergone serial passaging. We demonstrate that at serial passage six, the cells have greatly increased cellular senescence (as assessed by -galactosidase staining) and increased levels of DNA damage (as assessed by immunofluorescence with a -H2AX antibody). Importantly, we were able to successfully correlate those increases in cellular senescence and DNA damage to an increase in the levels of the miR-29 family. This new and to our view very relevant information has been included in Fig. 2 of the revised manuscript and discussed in page 11.

5. The authors refer to the down-regulation of miR-29 in Ames dwarf mice. This is an interesting observation that could be potentially examined in the context of this work as well. For instance, it makes one wonder whether down-regulation of miR-29 could potentially delay replicative senescence in the tested cell cultures employed in this study. Such experiments, however, can only be carried out once proper markers (gH2aX, b-galactosidase or other) have been thoroughly established in the aforementioned system.

We also agree that depleting levels of miR-29 to study the ensuing effects on senescence and DNA damage is a logical step based on the observation of low miR-29 levels in an animal model of longevity. As such, we attempted to delay replicative senescence in Zmpste24-/- fibroblasts by the transfection of antagomiR molecules against mir-29. Although we consistently observed an increase in proliferative potential in the anti-miR-29 transfected cell cultures, the results were not significant in comparison to scrambled anti-miR transfected cell lines. Our attempts to improve inhibition efficiency were exhaustive, with antagomiRs from two different suppliers (Dharmacon and Ambion) being employed, in addition to extensive experimental optimization attempts. The inability of antagomiRs to rescue the cells from senescence is disappointing but not unexpected as the inherent difficulties in ablating DNA damage in progeroid cell lines are well documented. This is exemplified by farnesyltransferase inhibitor treatment of Hutchinson-Gilford progeria syndrome and restrictive dermopathy fibroblasts in which the prelamin A accumulation and aberrant nuclear morphology is effectively corrected, but no reduction occurs in DNA damage (Liu et al., J. Cell Sci 2006). Further, additional independent physiological pathways contribute to accelerated aging in Zmpste24 -/- mice as confirmed by the incomplete reversal of disease phenotypes when crossed onto a p53-/- background (Varela et al., Nature 2005) as well as by our recent documentation of extensive metabolic changes in progeroid mice (Marino et al, PNAS 2010), which cause a series of systemic effects at the organismal level that are very difficult to recapitulate and eventually rescue in an in vitro system. Consistent with our proposal that this non-significant reversal of cellular senescence can be attributed to an inadequate level of inhibition by the antagomiRs just in these particular replicative senescence assays, it is remarkable that we have got positive results with these reagents in experiments aimed at analyzing the effect of miR-29 in the modulation of the DNA damage response through Ppm1d in fibroblasts (see Fig. 8, in the revised manuscript).
Taken together all these considerations, we have concluded that the antagonism of the substantial miR29 levels induced during cellular senescence that is necessary to provide a substantial reversal

of this phenotype is at present an experimental limitation. Critically, we have however been able to provide the reverse experiment as proof-of-concept for the role of miR-29 in cellular senescence. The transfection of wild-type primary murine fibroblasts with pre-miR-29 molecules was able to strongly reduce proliferation in these cells and to increase the amount of senescent cells in comparison to scrambled control transfected cells (see Fig. 7A-D, in the revised manuscript). To our view, these experiments emphasize that we have successfully been able to demonstrate the mechanistic link between elevated miR-29 levels and DNA damage-induced senescence. Finally, the relevance of this mechanism in the context of natural aging has received significant correlative support during the review process of our manuscript after careful analysis of data reported by Somet et al (Genome Res. 2010; Supplementary Tables), in which we have noticed that expression of miR-29 family members is significantly increased during aging in human and macaque brains.

6. Besides single and double strand breaks, Doxorubicin induces a potent G2/M arrest. How the authors exclude that the miR29 upregulation (compared to untreated wt cells) is not due to cell cycle differences (rather than DNA damage)? Were the untreated cells carried along with the treated ones (time vehicle control) during cell culturing or were the control cells taken at "zero" timepoint (before treatment)? This point should be clarified.

All control samples were carried through all experimental time points in parallel with the treated samples to ensure similar cell cycles were being assessed. As an additional control we analyzed the miR-29 family expression levels at the cell cycle checkpoints, G0, G1, G2 and M, finding no difference in expression. This question has been clarified in the revised manuscript (Figure legend 3).

7. Doxorubicin is used throughout the work as the sole DNA damage-inducing treatment. To strengthen the relevance of DNA damage to miR-29 upregulation the authors should try additional genotoxic agents (e.g. UV, mitomycin, 4NQO, H202). For instance, doxorubicin induces permanent lesions or lesions that are difficult to repair whereas H202 only induces transient ones that are rapidly and efficiently repaired. Would miR-29 upregulation be seen upon exposure of cells to both treatments? This is important to judge whether the up-regulation of miR-29 is relevant to lesions accumulating gradually upon natural aging or else rapidly upon progeria.

We acknowledge the significance of defining the type of DNA damage that induces miR-29 transcription. To adequately answer this question, we chose in addition to analyzing double stranded DNA breaks and complex lesion formation by doxorubicin, to examine free radical single stranded DNA breaks by H202 and UV-mimetic DNA damage by 4-nitroquinoline-1-oxide (4NQO). Interestingly, only doxorubicin induced the upregulation of the miR-29 family (as assessed by qPCR and luciferase assay) suggesting that chronic damage or difficult to repair lesions are the main factors inducing miR-29 transcription. Furthermore, this provides evidence to suggest that the genome maintenance mechanisms associated with double stranded break repair rather than nucleotide-excision repair or base-excision repair, underlies the regulation of the miR-29 family in response to DNA damage. This interesting aspect has been now discussed in the revised manuscript (Fig. 3 and page 12).

8. The authors show evidence that miR-29 is upregulated upon DNA damage and that the miR-29 family of miRNAs is implicated in the response to DNA damage. If these important claims are to be made, then the authors should also provide evidence that cells impaired in miR-29 or else treated with anti-miR29 are more sensitive to doxorubicin or g-irradiation (both induce double strand breaks). In addition, the authors could test the expression levels of miR-29 in cell lines defective in ATM (deals mainly with the response to DSBs) as opposed to ATR (deals mainly with the response to UV-induced transcription-blocking DNA lesions). Without such in vitro functional evidence, the conclusion(s) drawn in the manuscript are rather weakly justified.

To provide additional in vitro justification for the role of miR-29 in the induction of cellular senescence we have demonstrated that the transfection of wild-type primary fibroblasts with pre-miR-29 molecules was able to strongly sensitize the cells to doxorubicin-induced cellular senescence. In addition, on the advice of the reviewer, we examined the expression of the miR-29 family in cell lines from ATM- and ATR-deficient mice in comparison to their respective wild-type littermates, but we did not find any significant changes in miR-29 expression when cells from these

mutant mice were treated with 1 M doxorubicin. Only after forcing the experimental conditions and using high doses of doxorubicin (3 M) it was possible to detect significant increases in miR-29 levels. These experiments are shown in Suppl. Fig. 1C of the revised manuscript and have been discussed in pages 12 and 18.

9. Along the same lines, the physiological in vivo relevance of miR-29 to DNA damage is lacking. For instance the authors could test the expression of miR-29 in progeroid, DNA repair-deficient mice (e.g. NER or other). The advantage of examining the expression of miR-29 in tissues derived from DNA repair-deficient mice is that the relevance of miR-29 to DNA damage will then be examined upon intrinsic rather than exogenous genotoxic sources, thereby making it more relevant to the natural aging situation.

Following the reviewerís recommendation, we have also analyzed the putative changes in miR-29 expression levels in some available tissues from progeroid mice deficient in other genes implicated in DNA-repair (XPF, CSB/XPA, ATM and ATR-Seckel) but we have not found significant changes in any of them. Overall, these data would suggest that the miR-29 increase in the Zmpste24-/- model is dependent on a chronic DNA damage due to a very specific genotoxic stress caused by nuclear envelope dysfunction, but not initiated in response to intrinsic genomic stress derived from repair machinery impairment. These data have been discussed in page 10. Finally, and in relation to the relevance of miR-29 in a natural aging situation, we must emphasize again that our findings are supported by the very recent work from Somel et al, discussed above, correlating increasing levels of miR-29 in the cortex of aging human and macaque.

10. On the proposed model (page 17, last paragraph): The authors should take into consideration that Ames dwarf mice that show down-regulation of miR-29 are long-lived animals primarily due to the fact that they show very low tumor frequency. Isn't that result (i.e. the down-regulation of miR-29 in a tumor-free model) somehow contradictory to what the authors proposed in page 17, last paragraph (the potential role of miR29 in tumor predisposition)? If possible an additional explanation should be discussed.

This an interesting point and has identified an area of our discussion that could be further refined. We would argue that the Ames dwarf mice are in fact not a tumor-free-mode as they display very similar tumor frequency to wild-type animals; it is however their greatly increased tumor latency that is a distinguishing feature. Our hypothesis on page 17 of the previous manuscript is directed towards tumor cell behaviour as opposed to an involvement in tumorigenesis, which we readily agree needs clarification in the discussion. As such, the corresponding paragraph in page 18 of the revised manuscript has now been modified accordingly to clarify this question.

11. Indeed miR-29 appears to increase upon aging but the authors have not adequately clarified why. Do they assume it is due to gradual accumulation of DNA damage with advancing age? Perhaps a model figure could be generated that will discuss their proposed hypothesis in a rather more informative context (highlighting the primary instigator of the response and the relevant endpoints).

Our conclusion is that Zmpste24-progeroid mice demonstrate accelerated aging due to high levels of DNA damage that results from a perturbed DNA damage response. It is this chronic response to DNA damage induced by nuclear envelope defects that leads to the up-regulation of the miR-29 family, and their subsequent participation in cellular arrest. In order to ensure that this conclusion is clear in the revised manuscript, we have added a schematic diagram as Figure 9 that outlines our conclusions.

12. The chosen title is somewhat puzzling: Is it the DNA-damage response that activates a regulatory pathway involving miR-29 or the DNA damage itself?

In retrospect, we agree that the previous manuscript title is not totally adequate. To ensure that the central message of the paper is reflected by the title, the revised manuscript is now titled "Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53". This new title emphasizes the idea that it is the chronic response to DNA damage itself the mechanism responsible for the continuous activation of this regulatory pathway. We have also made an attempt

to specifically mention in the title that this mechanism has been uncovered in the context of nuclear envelope abnormalities characteristic of Zmpste24-null progeroid mice but also occurring during normal aging (see Scaffidi & Misteli, Science 2006). However, the strict limitations in the number of characters admitted in the title of EMBO J. papers, has made impossible the inclusion of this idea in the title of our revised manuscript.

Referee #2 (Remarks to the Author):
In this manuscript, Ugalde et al. start by analyzing miRNA species expression in wildtype and Zmpste24-deficient mice, which exhibit premature aging phenotypes. They have identified miR-29a, miR-29b, and miR-29c as a family of microRNAs that are consistently upregulated in liver and muscle of the Zmpste24-deficient mice and in old wildtype mice. They go on to show that miR-29s are upregulated in DNA damaged and late passaged MEFs in a p53-dependent manner. Using a number of bioinformatics tools they identify a number of potential miR-29 targets and confirm that these genes are indeed targeted in a luciferase-based assay. Particular attention as a mR-29 target is paid to Wip1, a phosphatase that regulates p53 and ATM signaling. The authors show in human cancer cell lines that miR-29 species do suppress Wip1 protein levels and presumably function as measured by phospho-p53 levels. The authors propose the existence of a novel regulatory circuitry involving miR-29, Ppm1d and p53, which is activated in aging and in response to DNA damage. This is a well written, very exciting paper which reveals a potential new microRNA-based aging/DNA damage-associated pathway and as such would be of general interest to EMBO Journal readers. While the data here is fairly convincing regarding this miR-29 pathway, the story could be made even more compelling if a few additional experiments could be performed.
1) First, the data showing that p53 regulates miR-29s is confined to Fig. 2C and is limited in nature. How does p53 upregulate miR-29s? Is it through direct transcriptional upregulation? Did the authors look for p53 RE consensus sites in the miR-29 promoter? What about miR-29 promoter-luciferase experiments with and without p53? Or a ChIP assay with p53 Ab on the miR-29 promoter?

We agree that delineating the involvement of p53 in the regulation of the miR-29 family is of great interest. To answer this, we have cloned the promoters of the miR-29 AB and BC clusters and performed a series of luciferase assays. Interestingly, and in agreement with qPCR experiments, activity of both clusterís promoters was strongly induced by doxorubicin, but not by H2O2 or 4-NQO in HCT116 cells. Further, by comparative analysis of the miR-29 AB and BC promoter activities in HCT116 wild-type and HCT116 p53-/- cell lines, we have determined that p53 is required for increases in promoter activity in response to doxorubicin, which is in agreement with qPCR experiments in p53-/- primary fibroblasts and is further supported by the observations made by Tarasov et al (Cell Cycle, 2007) that miR-29a is a direct transcriptional target for p53. This new information has been described in the revised manuscript (Fig 3, page 12).

2) Second, the finding that miR-29s suppress Wip1 protein levels in cancer cell lines is exciting. But what about in wildtype vs. Zmpste24-/- MEFs or in wildtype vs. Zmpste24-/- tissues? Such experiments would more directly show the relevance of this pathway in the premature aging phenotypes exhibited by the Zmpste24-/- mice.

We appreciate that the Wip1/Ppm1d data enclosed in the previous manuscript, while exciting, focused on cancerous human cell lines and the mechanism in the context of non-cancerous cell lines is lacking. We have performed the experiments that the reviewer has suggested, analyzing Ppm1d levels in wildtype vs. Zmpste24-/- MEFs and in wildtype vs. Zmpste24-/- tissues but have encountered a technical issue, specifically poor antibody specificity for mouse Ppm1d. We have used three different commercial antibodies towards this purpose (Abcam ab37495, Santacruz SC-20712 and Bethyl Laboratories A300-664A), with westerns not producing a reliable and reproducible profile for this protein in mouse cell lysates or tissue extracts. However, as we have shown previously, western-blot analysis for PPM1D in human cell lines is highly specific (Fig. 8). In order to ensure we make a link to premature aging, we have extended our prior observations of elevated miR-29 levels inducing p53 phosphorylation and PPM1D protein level decreases in the human cancer line U2OS, to primary mouse fibroblasts, with the limitation of showing PPM1D protein levels. However, we could clearly reproduce the results in p53 phosphorylation in this system. This new information has been included in Fig. 8 and discussed in page 15 of the revised

manuscript.

Other points:
(1) Figure 2A: Only miR-29b is shown. Were the results for miR-29a and miR-29c similar?
We have added data showing that miR-29a and miR-29b are similarly upregulated.

(2) Figure 5A, 5B: The gene being assayed (narf or PPM1D) should be indicated in the Figure.
This has been modified in the revised manuscript.

(3) Figure 6A: Wip1 levels in the untreated MCF-7 cells seem almost non-existent. Even unstressed MCF-7 cells normally have very high levels of Wip1. Are the authors confident about the quality of their Wip1 antibodies? Or is this just a short film exposure?

It was a short exposure to show the differences in the doxorubicin treated condition. However, this blot has been eliminated and replaced with much clearer observations (Fig. 8 in the revised manuscript).

(4) Figure 6C,D: Measuring Wip1 functional effects only by assaying phospho-p53 levels seems somewhat inadequate. The authors should at least include levels of p53 protein as well as phospho-p53 to see the relative levels of p53 stabilization as well as prove that phospho-p53 differences are due specifically to differences in phosphorylation and not p53 protein levels. Also, showing effects on phosphorylation levels of one or more other known Wip1 targets would be particularly compelling. Finally, Wip1 protein levels should also be shown in these panels to complete the story.

As requested, the western-blot studies in which we show Wip1/Ppm1d protein levels have now extended with the corresponding western analysis assessing protein levels of phosphorylated p53, total p53 and Ppm1d. Further, we have shown that in conditions of decreased Ppm1d protein levels, specifically pre-miR-29 transfected wild-type fibroblasts, we have increased levels of the Ppm1d phosphatase target, -H2AX. (see Figure 7C in the revised manuscript)

(5) Figure 5,6: Controls used for miR-29s were usually empty vectors or mutant miRs. Were antagomirs to miR-29s used to actually decrease miR-29 expression. Showing increased Wip1 functional activity with antagomirs would be particularly impressive.

We agree that the opposite observation in miR-29 functional experiments by using miRNA inhibitory molecules is an important point. First, in the previous manuscript we have shown that the inhibition of miR-29 by antagomiR molecules reverses the luciferase activity repression observed in HEK-293 cells transfected with the Ppm1d-luciferase reporter and treated with doxorubicin. Moreover, in the revised manuscript, we have shown a slight but consistent increase in the proliferation of Zmpste24-deficient fibroblasts transfected with antagomiR molecules. In addition, we have demonstrated that inhibition of miR-29 by the use of antagomiRs in mouse fibroblasts reduces p53 phosphorylation levels, although we have been unable to examine the putative correlation with Ppm1d protein levels due to antibody limitations. Likewise, we attempted to correlate human PPM1D levels with p53 phosphorylation in U2OS cells, but in this system the antagomiR strategy failed to increase PPM1D levels and thereby, p53 phosphorylation remains unchanged. We attribute this failure to putative changes in other miRNAs that could repress PPM1D and mask the miR-29 effect, or to the low levels of miR-29 in this human cell line (See Fig 8 in the revised manuscript)

Referee #3 (Remarks to the Author):
In this manuscript Ugalde et al. report upregulation of the miR-29 family of miRNAs in Zmpste24 -/- mice, a model of accelerated aging, and in physiologically aged mice. Upregulation of miR-29 miRNAs is associated with cellular senescence in vitro and is induced by the DNA damage agent Doxorubicin in a p53-dependent way. The authors identify the protein phosphatase Pmp1d as a target of the miR-29 miRNAs and show that increased levels of phosphorylated p53 are associated with ectopic expression of miR-29 miRNAs in U2OS cells. Based on these observations and their previous findings showing p53 signaling activation in Zmpste24 -/- mice, the authors propose that miR-29 miRNAs have a functional role in mediating the DNA damage response and the senescent phenotype in accelerated and physiological aging. The study addresses an interesting question.

miRNAs are being implicated in a growing number of cellular and physiological processes and very little is known about their role in the aging process. Zmpste24 -/- mice show a dramatic accelerating aging phenotype and represent a good model system to uncover mechanisms of aging. The upregulation of miR-29 in Zmpste24 -/- mice is clear, in particular in muscle, and the similarity with normally aged mice is remarkable. The fact that Ppm1d is a novel miR-29 target and that its upregulation upon DNA damage is inhibited by the microRNAs is also convincingly shown. However, these observations are circumstantial with respect to aging, and the functional link between miR-29, DNA damage, p53, Pmp1d, and aging is not clear. Most of the experiments are performed using artificial systems, human cell lines instead of Zmpste -/- cells, in which acute DNA damage is transiently induced and their physiological relevance is questionable. The study would be greatly improved by showing a functional effect of miR-29/ Pmp1d on the p53-mediated cellular senescence which characterizes Zmpste24 -/- mice. See below for more specific points. If such an effect cannot be convincingly shown all the conclusions about aging should be toned down and the manuscript should only focus on the link between miR-29/ Pmp1d and DNA damage.
Major points:
1) A functional link needs to be shown between miR-29 and aging (admittedly difficult) or at least cellular senesce. The authors have previously shown that Zmpste -/- MEFs show accelerated senescence (Varela et al. 2005) presumably due to high levels of DNA damage. What is the expression level of miR-29 miRNAs in Zmpste -/- MEFs over passages compared to wt MEFs (Fig.2a)? Also, and more importantly, can anti-miR-29 rescue the accelerated senescence of Zmpste -/- MEFs? The authors show many experiments involving ectopic expression of miR-29 but a convincing experiment showing a cellular effect of anti-miR-29 is needed to demonstrate a functional role of miR-29 upregulation in aging.

This is indeed a difficult link to effectively show, but an essential one, of which we provided a strong foundation for in the original manuscript. On the basis of the reviewerís many recommendations, the enclosed revised manuscript has been vastly improved in this respect. As stated by the reviewer, Zmpste24-/- tissues and MEFs demonstrate accelerated senescence due to high levels of DNA damage that results from a perturbed DNA damage response. In the revised manuscript, we have performed serial passaging experiments in wild-type and Zmpste24-/- primary fibroblasts and have demonstrated increased cellular senescence (as assessed by -galactosidase staining) and increased levels of DNA damage (as assessed by immunofluorescence with a -H2AX antibody) in both genotypes due to serial passaging (Figure 2A-E). Importantly, we were able to successfully correlate those increases in cellular senescence and DNA damage to an increase in the levels of the miR-29 family. Critically, the mechanistic link between elevated miR-29 levels and DNA damage-induced senescence were provided by experiments in which we transfected wild-type primary murine fibroblasts with pre-miR-29 molecules and observed strongly reduce proliferative potential due to increased senescence within the cell population (Figure 7A-D). Finally, and as discussed in our response to referee 1, the relevance of this mechanism in the context of natural aging has received significant support during the review process of our manuscript with the very recent publication of the work by Somet et al (Genome Res. 2010, Supplementary Tables) showing that miR-29 expression is significantly increased during ageing in human and macaque brains.

2) The model proposed by the author is that miR-29 miRNAs inhibit the phosphatase Pmp1d, thereby increasing p53-posphorylation and inducing a chronic DNA damage response. This, again, must be proven in a more relevant system than U2OS. The experiments shown in fig. 6C and 6D need be performed using wt and Zmpste24-/- MEFs and correlated with cellular senescence. Ectopic expression of miR-29 in wt cells is expected to increase p53-phosporylation and induce senescence while anti-miR-29 in Zmpste24-/- MEFs should have opposite effects.

This proposal has been substantially strengthened in the revised manuscript. Firstly, we have demonstrated that the exogenous elevation of miR-29 levels in wild-type primary fibroblasts causes significant increases in cellular senescence and a resulting reduction in proliferation. As outlined above, attempts to antagonize miR-29 in Zmpste24-/- primary fibroblasts and reverse cellular senescence were not successful, although we consistently observed an increase in proliferative potential in the anti-miR-29 transfected cell cultures. Secondly, we show that increasing levels of miR-29 strongly sensitized cells to DNA damage due to doxorubicin (assessed by increased levels of phosphorylated p53 in western blots) and serial passaging (as assessed by immunofluorescence with an anti -H2AX antibody) in wild-type mouse fibroblasts. To extend our observations, we have

attempted to analyze Wip1/Ppm1d levels in wild-type vs. Zmpste24-/- MEFs and in wildtype vs. Zmpste24-/- tissues, but the poor antibody specificity for mouse Ppm1d has prevented a conclusion being drawn. However, as we have shown previously, western blot analysis for human PPM1D in U2OS cells is highly specific. The introduction of pre-miR-29 into U2OS cells causes an increase in phosphorylated p53 and subsequent reduction in PPM1D levels under basal and doxorubicin stimulation. Further the introduction of antagomiRs against miR-29 reverses the accumulation of phosphorylated p53 in mouse fibroblasts (see Figure 8 in the revised manuscript).

3) Fig. 6C. The total levels of p53 need to be shown to demonstrate that miR-29 act indeed through Pmp1d and not by simply stabilizing p53 and increasing the protein levels as previously shown. Furthermore, the interpretation that miR-29 expressing cells show an exacerbated response to DNA damage and a delayed one to caffeine is questionable. The increase in p53 phosphorylation needs to be normalized to the basal levels (no doxorubicin), which are higher in miR-29 expressing cells. Upon normalization the response to DNA damage and caffeine is identical or even lower than the control.

Examining total levels of p53 is an excellent suggestion and was an unintended oversight in the original submission. In all situations where phosphorylated p53 has been examined, we have added the appropriate levels of total p53; showing that miR29 actions are through increased phosphorylated p53 levels and not the stabilization of the unphosphorylated form. We are somewhat confused by the reviewerís comments in regards to Figure 6C. Despite the relatively short period of treatment with caffeine (1 hour) following doxorubicin stimulation, we have provided a clear demonstration that caffeine is able to strongly reduce p53 phosphorylation due to miR29-sensitization to DNA damage. While there is some residual p53 phosphorylation in caffeine treated samples when compared to untreated samples, we would strongly argue that the conclusions we have drawn are well supported and as such we have maintained the same figure in the revised manuscript (Figure 8C) with the inclusion of total p53 levels.

Minor points:
1) The result showing that upregulation of miR-29 upon induction of DNA damage is abolished in p53 -/- cells is interesting. Do p53 -/- cells also fail to upregulate miR-29 over passages?

Following your suggestions, we have analyzed low passage vs. high passage mouse p53-/- primary fibroblasts, and we have not observed significant changes in miR-29 in these samples.

2) It is not clear why the authors use MCF7 cells to establish that Pmp1d is a target of miR-29 and then switch to U2OS cells for further validation of their hypothesis. In general, too many different cell lines are used in this study (MEFs, HEK293, MCF7 and U2OS) and this makes it hard to compare the different results.

We have rectified this situation using only mouse fibroblasts and U2OS for western blot analysis (Figure 8 in the revised manuscript). We acknowledge that the utilization of various cell lines in the previous manuscript made comparisons difficult, although it could be equally argued that confirmation of the mechanism in a range of cellular models strengthens the observation, rather than diminishing it.

3*) Fig. 6B. The claimed increase in Pmp1d levels in anti-mir expressing cells is not clear at all. A 1.2-fold increase detected by western blot (without standard deviation) cannot be trusted.*

This panel has been removed in the revised manuscript and replaced with an improved western blot (see Fig 8 in the revised manuscript).

4) The final model proposed by the authors is not clear. On one hand they seem to suggest that downregulation of Pmp1d by miR-29 leads to increased phosphorylation of p53 and this contributes to the activation a chronic, abnormally high, response to DNA damage and consequent senescence. On the other hand, in the discussion at p16 the authors speculate that reduced levels of Pmp1d sensitize Zmpste24 -/- cells to genotoxic stress, somehow independently of p53, and this in turn leads to activation of p53 signaling pathway. Please explain better.

We have modified the manuscript to clarify this point. Moreover, the model shown in Figure 9 will hopefully contribute to further clarify this question.

Finally, we want to thank the reviewers for their constructive comments and criticisms because by following their indications we have found our work considerably improved. We also want to mention that we have included Andrew J. Ramsay as a co-author of the revised manuscript because he has substantially contributed to perform many of the new experiments incorporated in the revised version of the paper. In the hope that this revised work can be now accepted for publication in The EMBO Journal.

2nd Editorial Decision 10 January 2011

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by all three original reviewers, who consider the manuscript significantly improved in response to the initial comments and thus in principle suited for The EMBO Journal. Nevertheless referee 3 retains a number of concerns regarding the conclusion on organismal aging, which would still need to be further clarified before acceptance, either through additional experimental insights (see suggestions in the report below) or more careful discussion and tempering of certain interpretations and statements in title and abstract.

I am therefore returning the study to you once more for a final round of revision, kindly inviting you to modify the manuscript in response to the referee comments detailed below.

Once we will have received your re-revised manuscript, we should then hopefully be able to proceed with its formal acceptance and publication.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors of the manuscript have addressed all 12 points of referee 1 in a concise and adequate manner. Thus, I recommend publication of the manuscript in EMBO J.

Referee #2 (Remarks to the Author):

The authors have been fairly responsive to all of the reviewers' comments, including this one (Reviewer #2). Although my two major points about p53 regulation and effects on Wip1 function in normal cells could have been more extensively addressed, I give the authors a lot of credit for performing additional clarifying experiments on these and other of my points. Since these points are not crucial to the basic themes of the paper, I have no major objections to the partial responses. Moreover, particularly impressive were the responses to Reviewer #1, where a number of new experiments were performed and new figure panels added. The manuscript has been considerably strengthened by these changes. This reviewer has no further major technical or conceptual issues with this paper.

Referee #3 (Remarks to the Author):

In the revised manuscript the authors have significantly improved their study and addressed many of the points that were raised. However, a few inconsistencies remain in key experiments, which make it difficult to reconcile some results. My major problem is about the significance of these findings with respect to the progeroid phenotype of Zmpste24 -/- mice. The only piece of data that links the prematurely aged mice to mir29 is the upregulation of the miRNA in the knock-out mice but all the functional experiments performed with Zmpste-/- MEFs have failed.

With reference to my previous major point 1, while it is true that a rescue of proliferation by anti-mir29 can be hard to achieve once the chronic response has been established, the authors could try to prevent senescence by infecting early passage MEFs with a lentiviral construct expressing the anti-mir29.
Even more unexpectedly, the levels of mir29, although increased over passages, do not correlate with accelerated senescence in Zmpste 24 -/- , since the levels are identical to wt cells at each passage. This result clearly indicates that mir29 does NOT play a major role in determining the accelerated senescence of Zmpste -/- cells and other factors are more important. On the other hand, the results showing increased senescence of wt fibroblasts upon overexpression of mir29 are convincing, suggesting that the model might be correct in some conditions (maybe physiological aging), whereas in lamin A-dependent accelerated aging the role of mir29 is not that important. I suggest the authors try:
1) to prevent accelerated senescence of Zmpste 24 -/- cells as mentioned above
2) to show at least that the increased p53-phosphorilation characteristic of UNTREATED Zmpste-/- MEFs can be decreased by antiMIR29, as shown in Fig 8 A using doxorubicin-treated wt MEFS (see previous major point 2)

If none of these experiments will provide the expected result, no conclusion about aging can be legitimately drawn based on the presented results and the manuscript should only focus on DNA damage and cellular senescence, with only speculations about (physiological) aging. Zmpste -/- mice would then only serve to justify why the authors focused on mir29, but the authors could not use their data to explain the progeroid phenotype of Zmpste -/- mice - both the text and Fig.9 would need to be extensively revised. As a matter of fact, Zmpste-/- cells/mice are only used in Fig 1 and 2 and the data in Fig. 2 should actually be deleted as they argue against the model. The whole study is based on wt cells and the authors should limit their conclusions to those cells.

Previous major point 3: I still do not understand what it means that the response to caffeine is delayed in mir29-expressing cells (p17). The difference between mir-29 and empty vector in the last 4 lanes of P-p53 (caffeine + doxorubicin - treated) and in the first 4 lanes (untreated) seems identical to me. Caffeine inhibits p53 phophorylation in the same way with or without mir29, the difference is just the basal level.

Previous minor point 1: I might have missed it but I cannot find where this result, which I think is quite important, is shown.

New minor point: the fact that mir29 overexpression increases DNA damage (fig.2C) is important as it adds a new layer of positive feedback: DNA damage induces mir29 which in turn further increases DNA damage. This should be added to the model in fig. 9.

2nd Revision - authors' response 28 March 2011

Please find enclosed a revised version of our manuscript entitled "Aging and chronic DNA damage response induced by nuclear lamina defects activate a regulatory pathway involving miR-29 and p53" (EMBOJ-2010-75490) by Ugalde et al. We are very grateful for the positive comments raised by reviewers #1 and #2. To directly address the additional concerns of reviewer #3, we have

performed a number of new experiments that have confirmed and extended our previous findings. A summary of our experimental findings in response to each of reviewer #3 concerns are provided below.

Referee #3 (Remarks to the Author):
1.- With reference to my previous major point 1, while it is true that a rescue of proliferation by anti-mir29 can be hard to achieve once the chronic response has been established, the authors could try to prevent senescence by infecting early passage MEFs with a lentiviral construct expressing the anti-mir29. Even more unexpectedly, the levels of mir29, although increased over passages, do not correlate with accelerated senescence in Zmpste 24 -/- , since the levels are identical to wt cells at each passage. This result clearly indicates that mir29 does NOT play a major role in determining the accelerated senescence of Zmpste -/- cells and other factors are more important. On the other hand, the results showing increased senescence of wt fibroblasts upon overexpression of mir29 are convincing, suggesting that the model might be correct in some conditions (maybe physiological aging), whereas in lamin A-dependent accelerated aging the role of mir29 is not that important. I suggest the authors try:1) to prevent accelerated senescence of Zmpste 24 -/- cells as mentioned above; 2) to show at least that the increased p53-phosphorilation characteristic of UNTREATED Zmpste-/- MEFs can be decreased by antiMIR29, as shown in Fig 8 A using doxorubicin-treated wt MEFS (see previous major point 2). If none of these experiments will provide the expected result, no conclusion about aging can be legitimately drawn based on the presented results and the manuscript should only focus on DNA damage and cellular senescence, with only speculations about (physiological) aging. Zmpste -/- mice would then only serve to justify why the authors focused on mir29, but the authors could not use their data to explain the progeroid phenotype of Zmpste -/- mice - both the text and Fig.9 would need to be extensively revised. As a matter of fact, Zmpste-/- cells/mice are only used in Fig 1 and 2 and the data in Fig. 2 should actually be deleted as they argue against the model. The whole study is based on wt cells and the authors should limit their conclusions to those cells.

We cannot dispute the reviewerís reservations regarding the failure of experiments aimed at preventing DNA damage-induced cellular senescence through miR-29 antagonism. As stated in our previous submission letter, we believed a primary cause for the failure of these experiments was unsatisfactory levels of inhibition by the employed antagomiRs. As such, we re-evaluated available approaches that could offer improved levels of miRNA inhibition. The reviewerís suggestion of using a lentiviral vector for miRNA inhibition was interesting, but the sole expression vector approach for miRNA delivery we could find, called miRNA sponges, have had inconsistent efficiency for several collaborators (personal communications). Consequently, we instead focused on the miRNA molecules themselves and utilized antagomiRs carrying a novel modification (LNA, locked nucleic acids) that extends their stability and increases the affinity for the target miRNA. These particular antagomiRs proved to be highly successful and have enabled us to perform additional experiments which have extended our previous findings. In summary of the outcomes from experiments employing LNA modified antagomiRs against miR-29, we have demonstrated that:

1. Transfection of the new inhibitors into passage 3 Zmpste24-/- primary fibroblasts greatly improves proliferation, as assessed by cell number counting over three passages (Figure 7B). As an alternative approach, and to be consistent with the experiments carried out in wild-type cells, we also performed MTT experiments with Zmpste24-/- fibroblasts transfected with the new inhibitor molecules, confirming improvement of proliferative potential upon miR-29 antagonism and demonstrating that treated cells display a modest but significant increase towards doxorubicin sensitivity (Supplementary Figure 2B).
2. Given the success of the new miRNA inhibitor molecules, we extended the proliferation analysis to the U2OS cell line. Thus, U2OS cells that stably over-express the cluster miR-29b-2~29c or cells carrying the empty vector were transfected with control or miR-29 inhibitor molecules and proliferation measured by MTT (Supplementary Figure 2C). As anticipated, U2OS cells expressing miR-29 showed a strong decrease in proliferation compared to cells carrying the empty vector, but more interestingly, this defect was completely corrected with transfection of miR-29 inhibitor molecules.
3. Building on the strong observations made in proliferation experiments, we were able to additionally confirm that LNA-modified antagomiRs against miR-29 when introduced into

Zmpste24-/- cells efficiently reduced cellular senescence (assessed by -galactosidase staining) and DNA damage response activation (assessed by -H2AX immunofluorescence). These results were in excellent accordance with our previous demonstrations of increased cellular senescence and DNA damage response when precursor miR-29 molecules were transfected into wild type cells.

2.- Previous major point 3: I still do not understand what it means that the response to caffeine is delayed in mir29-expressing cells (p17). The difference between mir-29 and empty vector in the last 4 lanes of P-p53 (caffeine + doxorubicin - treated) and in the first 4 lanes (untreated) seems identical to me. Caffeine inhibits p53 phosphorylation in the same way with or without mir29, the difference is just the basal level.

We feel this particular piece of data was unintentionally detracting from the manuscript. We agree with the reviewer that our conclusions were not clearly justified by the figure. In light of these particular results piece of data not being an essential element in our description of the DNA damage regulation of the miR-29 family, we have removed the caffeine regulation lanes of Figure 8D to avoid further confusion.

3.- Previous minor point 1: I might have missed it but I cannot find where this result, which I think is quite important, is shown.

In hindsight, we realize that we inadequately answered the reviewerís prior minor point 1. Our previous statement that p53-/- fibroblasts do not upregulate the miR-29 family over serial passaging was in fact a data not shown communication. This has been rectified and the data included as Supplementary Figure 2A of the revised manuscript and discussed in page 8 of the results section. As summarized previously, we were able to confirm that in the absence of p53, levels of miR-29 remain unchanged in primary fibroblasts over serial passages.

4.- New minor point: the fact that mir29 overexpression increases DNA damage (fig.2C) is important as it adds a new layer of positive feedback: DNA damage induces mir29 which in turn further increases DNA damage. This should be added to the model in fig. 9.

The reviewer has made an important point here which we agree needs to be highlighted in Figure 9. While it is clear from our results that miR-29 is increased in response to DNA damage and participates in the activation of the DNA damage pathway, the increase in -H2AX levels in response to elevated miR-29 expression could indeed represent additional DNA damage, concomitant with the identified increase in activation of the DNA damage response pathway. To acknowledge this intriguing potential positive feedback mechanism of miR-29 on DNA damage, we have altered Figure 9 as indicated by the reviewer and briefly discussed this point in page 17 of the revised manuscript.

Finally, we want to acknowledge again all these additional comments raised by reviewer #3, because by following his/her indications we have found our work considerably improved.

Acceptance letter 30 March 2011

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.

Yours sincerely,

Hartmut Vodermaier, PhD
Editor
The EMBO Journal

