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Oncogene-Induced Telomere Dysfunction Enforces Cellular Senescence in Human Cancer Precursor Lesions

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1st Editorial Decision 11 November 2011

Thank you very much for submitting your research manuscript that proposes telomere dysfunction-induced senescence as potential tumor suppressive mechanism in human cancer for consideration to The EMBO Journal editorial office.

I do apologize for some delay with the decision caused by relatively late comments from some of the referees. As you will see from their remarks, there is certainly interest though conditioned further conclusive experimentation that should also be aimed at elucidating underlying molecular mechanisms. It would thus need corroboration that various oncogenes trigger similar telomere replication defects (ref#1). Critical would also be a satisfying response to the question (raised by all refs), whether indeed oncogene-induced replication fork stalling rather than telomere shortening induced senescence that impairs tumor formation. Finally, refs#2 and #3 demand some further mechanistic understanding on telomerase rescue of oncogene-induced replication fork stalling and offer constructive suggestions on how to address this. Though relatively demanding and involving significant further experimentation, we decided to offer you the chance to thoroughly revise the current dataset. I urge you to take the referee comments serious and expense the necessary amount of time and efforts to avoid disappointments much later in the peer-review process.

I do also have to remind you that it is EMBO_J policy to allow a single round of revisions only and that the ultimate decision will depend on the stringency how these questions have been resolved

I am very much looking forward to your revised manuscript and remain with best regards

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The manuscript is divided in two parts. The first one, using clinical samples, shows that in various pre-cancerous lesions (skin, breast and colon samples) DDR activation and senescence correlates with telomere dysfunction. The second part provides evidence that oncogene-induced senescence leads to replication stress at telomeres leading to shortening.

The data of the first part are very convincing and clearly open new avenues in our understanding of the role of telomeres in human cancer. However, this referee is not convinced by the arguments of the authors that the telomere damages triggered in vitro by Ras overexpression is indeed the cause or even related to the increased rate of TIF observed in pre-cancerous lesions. For instance, as it seems to be the case from the submitted figures (see belmow), it seems difficult to conciliate the observation that dysfunctional telomeres in pre-cancerous lesions have the same length than the other telomeres of the cells while, in vitro, Ras induces marked telomere shortening events. Moreover, the cell types and the type of oncogene used in vitro are not relevant for the analyzed clinical samples. Alternative explanations of the clinical observation may be related to alterations in the expression of shelterin components (see Augereau et al, Blood, 2011).

Specifically, the major points are :

- what are the cells used in Figure 3 ? I guess from the fact that this technique requires very long telomeres and indication in the supplementary figures that they are mouse cells, i.e. with telomeres much longer than in human cells (normal or cancerous). Thus, what is the relevance of using this type of cells for the lesions studied in Figures 1 and 2 ?

- In order to really conclude that "Our data therefore demonstrate that telomeres are preferential sites of oncogene-induced DNA replication stress." , data comparing fork arrest at telomeres and at fragile sites must be shown.

- In melanoma, the main oncogene is mutated B-Raf. Therefore, it is critical to show that BRAFV600E triggers the same problem of telomere replication defect in melanocytes than RasV12 in fibroblasts, in particular TIF formation and replication defect.

- From the images shown in figure 3 and in supplementary figures, it seems that the PNA staining intensity of TIF+ and TIF- cells is similar. If this is correct, this would be in disfavor of the hypothesis that the recruitment of DDR proteins at telomeres is due to the shortening of telomeres induced in vitro by Ras overexpression. In any case, it is very important to show data comparing the PNA intensity of the telomeres colocalizing with 53BP1 with those not colocalizing ??

-The fact that hTERT overexpression protects against OIS does not necessarily mean that it is the catalytic function of telomerase (and thus telomere length) that is involved. Telomerase is an auxiliary factor of the Wnt signaling pathway and this signaling is also involved in senescence regulation. It is important to show that the effect of hTERT overexpression depends upon its catalytic activity (by overexpressing catalytically-dead alleles).

- the observation that Ras-induced senescent cells exhibit an increase rate of telomere dysfunction with time is interesting but the underlying mechanisms are quite obscure. Therefore, this result appears preliminary and difficult to interpret.

Minor points :
- in the introduction and discussion sections, it should be fair to cite Augereau et al (Blood, 2011) showing for the first time that early stages of a human malignancy (B-cell chronic leukemia) correlate with an increased rate of telomere dysfunction (measured by TIF) associated to a senescent-like phenotype (G0 arrest, p16 activation).

- Regarding the capacity of a single or limited number of telomeres can trigger senescence, it would be fair to cite Hemann et al, Cell, 2001 and Abdallah et al, NCB 2009.

- ´ ....and telomeres resemble fragile sites (Martinez et al, 2009; Sfeir et al, 2009) [a] it would be fair to also cite Ye et al, Cell, 2010.

Referee #2:

Suram et al. investigate telomere dysfunction induced senescence in human carcinogenesis. The authors show evidence for telomere dysfunction induced DNA damage foci (TIFs) in ealy stages of 3 different types of human tumors (melanoma, breast, colon). Interestingly, one of the tumor types (melanoma) shows long telomeres yet the tumors display TIFs. The authors go on and show that oncogene activation (H-RasV12) induces replication stress and replication fork stalling at the telomere eliciting a DNA damage response reminiscent to telomere dysfunction induced senescence. The authors also present data showing that telomerase expression can rescue the activation of DNA damage signals at telomeres in context of oncogene activation. The authors propose that tumor suppression by telomeres involves oncogene induced TIFs rather than the classical model proposing that telomere shortening impairs clonal expansion of telomeres.

The authors present an interesting idea and some experimental data supporting their hypothesis. However, there are some concerns that need to be addressed:

1. Is it really oncogene induced replication fork stalling in telomeres that impairs tumor formation in humans as opposed to telomere shortening induced senescence. This question remains debatable, especially since the authors investigated two tumor types (breast and colon) that often exhibit very short telomeres at early stages of carcinogenesis. It remains possible that replication fork stalling represent a side effect of in vivo oncogene activation, which is not sufficient to induce senescence. Instead a small number of 'true' dysfunctional telomeres (lacking telomeric sequences) may be responsible for senescence induction. This concern is even more important given that studies from Taylor Jacks have shown that endogenous activation of oncogenes is not leading to induction of senescence, which appears only in response to over expression of oncogenes at high levels in cell culture (Cancer Cell 2004).
2. How can telomerase rescue oncogene induced replication fork stalling? The classical role of telomerase is to add telomere repeats to chromosomal ends. How could that help to resuce a stalled replication fork? Isn't it still more plausible that the stalled forks only represent a a side effect but the true senescence inducing mechanism remains telomere dysfunction induced by loss of telomere repeats, which can be rescued by telomerase activation. This concern is strengthened by the observation that tumor formation is not significantly reduced in first generation of telomerase knockout mice (see papers of DePinho, Chin and Greenberg). First generation telomerase knockout mice have sufficient telomere repeats. If oncogene induced replication fork stalling is the main mechanism of tumor suppression in telomerase negative cells, then these mice should be well protected. However, this is not the case, but telomerase knockout mice show tumor suppression only in later generations when telomeres are critically short. These data indicate that telomere shortening rather then oncogene induced telomere dysfunction is most important in tumor suppression.
3. The observation that oncogene induced senescence is not stable in TERT expressing cells is interesting. It stands in contrast with previous publications from the Weinberg lab. The authors should demonstrate a mechanism how telomerase expression can revert oncogene induced stress. Is it really a rescue in replication fork stalling? Or are other mechanisms involved, e.g. ROS? Wnt-signalling? Telomere dysfunction induced by shortening?

Referee #3:

Activation of cellular senescence programs in mouse models of cancer has demonstrated that dysfunctional telomere induced cellular senescence is as potent as apoptosis in terms of tumor suppression. However, convincing examples of dysfunctional telomere-induced senescence

inhibiting the onset of human cancers are lacking. In this submission, Suram et al show that TDIS (telomere dysfunction- induced senescence) is indeed a tumor suppression mechanism in humans, using human cancer sample and human fibroblast expressing oncogenic Ras. They found that cancer precursor lesions of the skin, colon and breast but not frank malignancies display TDIS. The authors suggest that telomeres are sensitive to oncogene-induced replication stress, which results in telomere shortening and telomere dysfunction. Therefore, TDIS mediated by oncogene-induced DNA replication stress is a potentially important tumor suppressing mechanism. In addition, the authors propose that increased hTERT activity abrogates Ras- induced telomeric DDR foci formation, thereby preventing oncogene induced senescence (and TDIS).

Although the data is exciting and largely convincing, additional mechanistic insights will improve the manuscript. These authors have previously shown that oncogene induces replication stress promotes a DNA damage response, leading to checkpoint activation and the onset of cellular senescence. Therefore, the most interesting part of this study is understanding how TDIS occurs in precursor lesions. For example, what is the status of telomerase activity and/or hTERT expression in precursor lesion vs. malignant cancer? Could the authors correlate decreased hTERT activity in cancer precursor lesions with an increase in TDIS?

One problem with this study is the well known observation that telomerase expression is actually elevated in advanced malignancies. If the authors are correct, increased telomerase expression should quell TDIS, thereby eliminating this driver of genomic instability.

Other points:
Considering the role of hTERT in preventing OIS, could overexpression of hTERT abrogate Ras-induced telomeric replication stress? Data from replication experiment (Figure 3) using cells with both hTERT and Ras will be helpful.

Does BrdU incorporation rate increase in cells with Ras- hTERT at day29 compared to Ras expressing cells? Additional BrdU data from day29 group in Figure 5B will be more informative than data from day14 alone.

Figure 5D needs to indicate statistical significance properly. Especially, a comparison between the Ras-Ev and Ras-hTERT groups at 29 days is necessary.

To distinguish non-telomeric DDR and telomeric DDR in Figure 6D, co-immunostaining with telomere (PNA) and pATR will be needed.

Minor comments
The figures need more details. Although the figure legends indicate details, the figures should explain the experiment as well. Immunostaining result in Figure 1D and Figure 2D has to specify for which protein is being stained.

In figure 5E, what does the y-axis indicate? The y-axis needs to indicate whether the percentage is normalized to total cells or 53BP1 positive cells

1st Revision - Authors' Response 21 March 2012

We thank the reviewers for their useful comments. Based on these comments, we have now conducted a significant number of additional experiments which greatly improved the quality of this study. We have additionally included:

1) results demonstrating that dysfunctional telomeres in cancer precursor lesions and in oncogene expressing cells are not among the shortest telomeres when compared to other telomeres in the same cells (Figure 1E and Supplementary Figures S5B, S5D, S9D). Therefore, DDR activation at telomeres in cells of cancer precursor lesions is not (exclusively) a consequence of ***critical*** telomere attrition.

2) results demonstrating aberrant telomeric structures resembling fragile telomeres in TIF of cancer precursor lesions (Figure 1E and Supplementary Figures S2F, S5B). This provides further evidence that telomere dysfunction in human cancer precursor lesions is a consequence of telomeric replication stress.
3) results demonstrating that TRF2 is retained at telomeric DDR foci in cells of nevi (Supplementary Figure S2E). Therefore, telomere dysfunction in these cancer precursor lesions is not a consequence of TRF2 loss.
4) results demonstrating that oncogenic Ras has more modest impact on DNA replication at non telomeric sequences (Supplementary Figure S7B). Therefore, telomeres are preferential sites of oncogene induced DNA replication for stalling.
5) results demonstrating that hTERT expression does not rescue the fragile telomere phenotype induced by oncogenic Ras (Supplementary Figure S8A). This is consistent with our data demonstrating that Ras causes telomeric replication fork stalling even in TERT positive MEFs. Our new data therefore confirm that hTERT does not eliminate oncogene induced-telomeric replication stress.
6) results demonstrating that hTERT expression prevents stochastic telomere erosion in oncogene expressing cells (Supplementary Figure S8D).
7) results demonstrating that the catalytic activity of hTERT is required to destabilize RasV12 induced cellular senescence (Supplementary Figure S9F-H). Together, our data therefore suggest that telomerase counteracts oncogene induced-telomere dysfunction by mechanisms that resemble (or are identical to) telomere healing.
8) results demonstrating that another oncogene, B-RafV600E (an oncogenic mutation that is found in melanocytic cells of most nevi) also causes telomeric replication stress, telomere dysfunction, and consequently cellular senescence in normal human cells. Similar to Ras induced senescence that is destabilized by hTERT expression, BRafV600E induced-senescence is ***not*** stable in hTERT expressing cells (Supplementary Figure S10A-E). Therefore, telomere dysfunction enforces cellular senescence triggered also by oncogenes other than H-RasV12.

We have incorporated these new data into the manuscript and show newly added text in red for easier identification. Below, we respond to the individual comments.

Reviewer #1:

The manuscript is divided in two parts. The first one, using clinical samples, shows that in various pre-cancerous lesions (skin, breast and colon samples) DDR activation and senescence correlates with telomere dysfunction. The second part provides evidence that oncogene-induced senescence leads to replication stress at telomeres leading to shortening. The data of the first part are very convincing and clearly open new avenues in our understanding of the role of telomeres in human cancer. However, this referee is not convinced by the arguments of the authors that the telomere damages triggered in vitro by Ras overexpression is indeed the cause or even related to the increased rate of TIF observed in pre-cancerous lesions. For instance, as it seems to be the case from the submitted figures (see below), it seems difficult to conciliate the observation that dysfunctional telomeres in pre-cancerous lesions have the same length than the other telomeres of the cells while, in vitro, Ras induces marked telomere shortening events. Moreover, the cell types and the type of oncogene used in vitro are not relevant for the analyzed clinical samples. Alternative explanations of the clinical observation may be related to alterations in the expression of shelterin components (see Augereau et al, Blood, 2011).

Specifically, the major points are :

- what are the cells used in Figure 3 ? I guess from the fact that this technique requires very long telomeres and indication in the supplementary figures that they are mouse cells, i.e. with telomeres much longer than in human cells (normal or cancerous). Thus, what is the relevance of using this type of cells for the lesions studied in Figures 1 and 2?

ANSWER 1) The referee indeed is correct in understanding that mouse cells were used for the experiments in Figure 3. We had stated this in the figure legend, but now also specifically mention this in the Materials and Methods in order to avoid any confusion. The reason why we used specifically this cell type for our analysis is because telomere lengths in these cells are approximately 4-10 fold longer compared to telomeres in human cells.

Due to the short nature of human telomeres (~8-10kb), it is not feasible to perform telomeric DNA combing using cells from humans. Mouse cells contain telomeres that are dramatically longer compared to human telomeres (~40-80kb). For this reason, we used mouse embryonic fibroblasts, so far the only cell type that has been used successfully to perform telomeric DNA combing (Sfeir et al., 2009 Cell *138* 90). We believe that the primary reason why not a single study has been published to date demonstrating the feasibility of telomeric DNA combing using cells other that MEFs is because telomeres in other species are too short to be visualized on stretched DNA fibers.

REVIEWER: In order to really conclude that "Our data therefore demonstrate that telomeres are preferential sites of oncogene-induced DNA replication stress." , data comparing fork arrest at telomeres and at fragile sites must be shown.

ANSWER 2) Because of the reasons stated above, we used mouse cells to characterize replication fork stalling events in telomeric repeats. In this organism, however, fragile sites have not been mapped as thoroughly compared to human cells. Therefore, it is currently not possible to effectively compare telomeric replication fork stalling events to those that occur specifically at other fragile sites using mouse cells. However, we have now additionally conducted an analysis to determine whether DNA replication forks stall at greater rates in telomeric repeats compared to non telomeric sequences in response to oncogenic signals. In our initial submission, we demonstrated a statistically highly significant increase in telomere replication fork stalling events in Ras expressing cells when compared to control empty vector expressing cells (p=0.03). The experimental set up we use to study DNA replication dynamics at telomeres was specifically designed to study these regions only – we use repeated long (1 hour) pulses with thymdine analogs (as also used in Sfeir et al. 2009 Cell *138* 90) and is not best suited to highlight whole genome DNA replication pattern differences. Nevertheless, following this referee's request, we scored the symmetry of 41 completely random and ***non telomeric replication forks*** in both Ras and empty vector expressing cells and plotted the symmetry of replication forks (1 = complete symmetry; 0 = total asymmetry) for each fork analyzed. This type of analysis illustrates that, compared to control cells, Ras also causes DNA replication fork stalling events in non-telomeric sequences (see Figure S7B). However, the differences were statistically not significant for the technical reasons explained above, while telomeric-only replication fork stalling events (see Figure 3D; p=0.03). Therefore, we can conclude from this analysis that oncogenic Ras makes a stronger impact on telomeric DNA sequences compared to non telomeric ones, which show nevertheless a modest (under the conditions employed) but detectable sensitivity to oncogene expression.

Since our initial conclusion might have been too ambiguous, as indicated by the referee, we have now changed the sentence to "our data therefore demonstrate that telomeres are preferential sites of oncogene-induced DNA replication stress when compared to the rest of the genome."

REVIEWER: In melanoma, the main oncogene is mutated B-Raf. Therefore, it is critical to show that BRAFV600E triggers the same problem of telomere replication defect in melanocytes than RasV12 in fibroblasts, in particular TIF formation and replication defect.

ANSWER 3) We thank the reviewer for suggesting these important experiments. In fact, we have been in communication with Dr. Dorothy Bennett in regards to conducting exactly these experiments and acquiring human melanocytes from her laboratory. Unfortunately, conducting exactly these experiments -BRafV600E expression in human melanocytes that either lack hTERT or express hTERT- is not feasible for the following reasons:

i) Human melanocytes (including neonatal melanocytes) are notoriously difficult to maintain and manipulate in culture. They cannot be "immortalized" using hTERT even in the presence of feeder cells, unless p16INK4a is inactivated (Sviderskaya et al., 2003 J Natl Cancer Inst **95** p723). This problem is also encountered by other cell types, such as human mammary epithelial cells and human ***lung*** fibroblasts and has been attributed to inadequate culture conditions that cause upregulation of p16 and consequently premature cellular senescence (for example see Forsyth et al., 2003 Aging Cell **2** p235).

ii) expression of BRafV600 in cultured human melanocytes at levels similar to those found in nevi, causes rapid cellular senescence as a consequence of p16INK4a upregulation (Michaloglou et al., 2005 Nature **436** p720; Gray-Schopfer et al., 2006 **95** p496). At the single cell level, p16 protein levels are highly elevated in every single cell (same references and our unpublished data). Furthermore, melanocytes almost immediately cease proliferation following BRafV600E expression. As evident from these published data, cells expressing BRafV600E do not appear to proliferate at all (see references above). In fact, cell number appears to decrease slightly in melanocyte cultures that express oncogenic BRaf suggesting that this oncogene also causes some apoptosis in these cells. This scenario may not be physiological given that nevi harboring the BRafV600E oncogene are comprised of cells that have undergone many population doublings. Furthermore, most nevi are comprised of senescent cells that often display heterogenous and mosaic p16 expression patterns. Some nevi even completely lack p16 expression, although cells display features of cellular senescence (same references). Therefore, senescence in cells of nevi is not only a consequence of p16 upregulation. This, however, is in contrast to cultured melanocytes that, as a first line of defense, activate the p16 senescence pathway upon BRafV600E expression.

One likely reason why BRafV600E induced melanocyte senescence in culture primarily activates the p16 senescence pathway is because culture stresses combined with oncogenic stresses rapidly upregulate p16, thereby preventing any significant proliferation. We have now also tested this experimentally. After transduction of BRafV600E into normal neonatal human melanocytes, followed by puromycin selection, cells did not proliferate significantly and rapidly entered cellular senescence. All cells displayed high levels of p16. However, since we cannot accumulate sufficient numbers of cells that are stably expressing BRafV600E (due to inherently inefficient transduction efficiencies of melanocytes and almost immediate cessation of growth), we are unable to measure telomeric replication defects in these cells. Furthermore, since normal human neonatal melanocytes cannot be "immortalized" with hTERT, the effects of telomerase on suppressing the BRafV600E induced telomeric DDR is not possible in this cell type.

Due to these limitations of human neonatal melanocytes, we now instead used human skin fibroblasts (BJ cells), which are highly resistant to culture shock induced p16 upregulation. BRafV600E was expressed stably in BJ cells lacking hTERT and, as a control, stably expressing hTERT. We have added these new data to our manuscript and show them in Supplemental Figure S10. Significantly, our results obtained with BRafV600E virtually mirror those obtained with H-RasV12. We demonstrate that BRafV600E triggers a permanent cellular growth arrest in BJ cells, and a transient proliferative arrest in BJ cells expressing hTERT. We show that the transient growth arrest in BJ-hTERT cells is not due to loss of BRafV600E expression. In normal fibroblasts lacking hTERT, BRafV600E caused transient non-telomeric DDR foci and persistent telomeric DDR foci. BRafV600E also caused fragile telomeres demonstrating that also this oncogene affects telomere replication. Telomerase positive cells did not display telomeric DDR foci demonstrating that hTERT suppresses telomere dysfunction in response to BRafV600E signaling. In conclusion, our new data demonstrate that multiple oncogenes, including the oncogene that is primarily associated with hyperproliferation in cells of nevi, affect telomere replication leading to telomere dysfunction and cellular senescence.

REVIEWER: From the images shown in figure 3 and in supplementary figures, it seems that the PNA staining intensity of TIF+ and TIF- cells is similar. If this is correct, this would be in disfavor

of the hypothesis that the recruitment of DDR proteins at telomeres is due to the shortening of telomeres induced in vitro by Ras overexpression.

ANSWER 4) We do not favor the model that that total telomere shortening is a requirement for telomere dysfunction induced cellular senescence. In addition, although complete erosion of a single telomere (also called here critical telomere shortening) will likely result in telomere dysfunction, we and our collaborators have strong evidence that critical telomere shortening is not the primary cause of telomere dysfunction in mammalian cells. For reasons outlined below we believe that it is primarily the formation of telomeric lesions such as double stranded DNA breaks (DSBs) that triggers telomere dysfunction and cellular senescence, both in cultured cells encountering DNA replication stress and in tissues, including cancer precursor lesions. Under certain conditions, such as during the serial passaging of somatic cells or as a consequence of RasV12 overexpresion, telomere dysfunction is associated with total and/or stochastic telomere erosion in the same cells. Other conditions, such as those outlined below, cause telomere dysfunction and cellular senescence in the absence of (or with undetectable) total telomere erosion. Variables that likely determine whether cells undergo TDIS with long or with short telomeres are cell type, type of genotoxic stress, and the levels and duration of these stresses. We mention this to a certain degree also in the discussion of our manuscript. However, we previously did not emphasize that telomeric DSBs are the cause of cellular senescence, as our collaborator Dr. Fabrizion d'Adda di Fagagna previously submitted a manuscript entitled "Telomeric DNA damage is irreparable and causes persistent DNA damage response activation" to *Nature Cell Biology* detailing exactly this conclusion (see below). This manuscript is now accepted for publication, and we have modified the text in our conclusions accordingly, referring to this paper and to the irreparability of telomeric lesions.

While our data demonstrate that oncogenic H-RasV12 also causes accelerated erosion of telomeres when lengths of all telomeres are considered, we believe that it is the formation of Ras-induced lesions such as a stalled telomeric replication fork or DSBs in telomeric repeats that causes telomere dysfunction. It is likely that a telomeric DSB also erodes the telomere in which the break has occurred (since the telomere portion not attached to the chromosome would be lost) thereby contributing to the observed loss of total telomere lengths in RasV12 expressing cells.

For the purpose of clarification, we call a telomere "dysfunctional" when we observe a colocalization between a telomeric signal (detected using antibodies against telomere binding proteins or a labeled peptide nucleic acid (PNA) complementary to telomeric repeats) and a DDR focus. This has also been called a telomere dysfunction induced focus or TIF by the de Lange lab. TIF are generated by different mechanisms. Originally, TIF were discovered in cells that over-express a dominant negative mutant of TRF2 and in cells depleted of TRF2 by siRNA (Takai et al., 2003 Current Biology). Telomeres in these cells were still long, including the dysfunctional telomeres, demonstrating that telomeres do not have to be critically short in order to become dysfunctional.TRF2 is absent in these TIF, demonstrating that TIF can be generated by removing TRF2 from the telomeres. We have now added data to this manuscript showing that TRF2 is still present in TIF of nevi. Therefore, it is not loss of TRF2 from the telomere that is responsible for generating dysfunctional telomeres in tissue (see Supplemental Figure S3E). Following the publication of the first manuscript describing TIF by the de Lange lab, we and others demonstrated that TIF can also be generated by the cell aging process, which is associated with shortening of total telomere lengths (Herbig et al., 2004 Mol Cell). Surprisingly, we discovered that most dysfunctional telomeres still contained sufficient telomeric repeats in order to be detected by labeled peptide nucleic acids complementary to telomeric repeats. While we did not analyze telomere lengths in TIF quantitatively, our data (that now have been reproduced numerous times) demonstrated that dysfunctional telomeres, also in the setting of total telomere erosion caused by continuing cell divisions, likely still contain kilobases of telomeric repeats. We published similar observations for dermal fibroblasts that accumulate exponentially in the skin of aging baboons. Also in these cells, dysfunctional telomeres still contained detectable telomeric repeats, and even sometimes displayed stronger telomeric signals compared to other telomeres in the same cell (Herbig et al., 2006 *Science*). We and our collaborators in the d'Adda di Fagagna lab have now demonstrated this also in other non-proliferating tissue of aging baboons (Fumagalli et al., 2012 *Nature Cell Biology*, in press;

doi:10.1038/ncb2466). These data suggest that also during normal aging, dysfunctional telomeres do not have to be critically short and completely eroded.

Finally, as mentioned above, we are collaborating with Dr. Fabrizio d'Adda di Fagagna whose group demonstrated that telomeric DSBs caused by DNA damaging agents, such as ionizing radiation, drugs, and site specific endonucleases are not repaired. As a result these telomeres are dysfunctional and consequently trigger cellular senescence. Using a modified Q-FISH protocol, his group also demonstrated that these dysfunctional telomeres generally are not shorter compared to non-dysfunctional telomeres in the same cells. Similarly, his group demonstrated that TIF positive cells are found in greater abundance in the hippocampus and liver of old baboons, compared to young animals, and also in these cells the dysfunctional telomeres were generally not shorter compared to other telomeres in the same cell/tissue. ***Thus, telomeres do not have to be critically short in order to become dysfunctional*** (Fumagalli et al. 2012. Telomeric DNA damage is irreparable and causes persistent DNA damage response activation. *Nature Cell Biology* doi:10.1038/ncb2466). A similar set of results from the Passos lab has just been published (Hewitt et al., Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. 2012 *Nature Communications* **3** p708).

REVIEWER: In any case, it is very important to show data comparing the PNA intensity of the telomeres colocalizing with 53BP1 with those not colocalizing ??

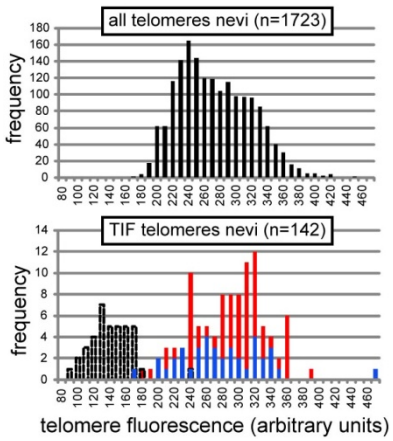
ANSWER 5) While this is not a concern of the reviewer, we nevertheless would like to mention here that it has been well established that total telomere lengths in cells of epithelial cancer precursor lesions, such as those in tubular adenomas are significantly shorter compared to total telomere lengths in normal epithelium or in surrounding stromal cells (for example see Meeker AK. 2004 Clin Cancer Res. **10** 3317). Telomeres in cells of ductal hyperplasias (DHs) generally display intermediate lengths (between normal epithelium and DCIS/IDC; Meeker AK et al., 2004 Am. J. Pathol), while total telomere lengths in cells of melanocytic nevi are similar to those in surrounding stromal cells (Michaloglou et al. 2005 Nature **436** 720). Together with our data demonstrating TIF in all of these cancer precursor lesions, it is unlikely that total telomere length measurements are useful for determining whether telomeres in cells of tissue are dysfunctional or not.

Therefore, if total telomere lengths do not predict whether a cell has a dysfunctional telomere in cancer precursor lesions, then short telomeres are likely not the primary cause for telomere dysfunction in tissue. What then could cause telomere dysfunction in these cells? Is it critical shortening of one or few telomeres in a cell with otherwise long telomeres, or the formation of telomeric lesions, potentially due to DNA replication stress? To address this, and to comply with the reviewers request, we now have applied the above mentioned modified telomere Q-FISH protocol and compared telomere signal intensities of telomeres in TIF to those not associated with DDR foci (non-TIF telomeres) in the same cells. We analyzed telomere lengths of cells in nevi, ductal breast hyperplasias, tubular adenomas, and Ras senescent cells and include these data in Figure 1E, and Supplemental Figures 5B, 5D, and 9D. We discovered that, similar to our collaborators' data, telomeres in TIF are generally not shorter compared to non-TIF telomeres in the same cells (for example see Figure below, TIF telomeres illustrated as red bars). These data therefore demonstrate that it is not critical shortening of individual telomeres that dictates whether the telomere is sensed as a DSB in cells of analyzed cancer precursor lesions and in oncogene expressing cells. Since dysfunctional telomeres are of similar lengths compared the other telomeres in the same cell, it is more likely that it is the formation of an irreparable telomeric lesion such as a DSB that causes telomere dysfunction in these cells.

Upon conducting telomere length analysis of cells in tissue we noticed that a significant percentage of telomeric signals in TIF appeared as doublets. In nevi, 28% of telomeres in TIF appeared as doublets, while in DHs 8% of telomeres appeared as doublets. Doublets were not apparent in TIF of cells in tubular adenomas, likely due to the fact that almost all telomeres in these cells are so short that aberrant telomeric structures fall below the detection limit when using fluorescence microscopy.

Representative images illustrating these doublets in cells of nevi and DH's are shown in Supplemental Figure S2F and S5B and can also readily be observed in TIF micrographs of our initial submission -Figure 1C- although initially we did not discuss them). Since the software used to quantitate telomere signal intensities often could not discriminate between the two foci, we measured combined signal intensities of the doublets which placed the combined signal intensities of these telomeric doublets (blue bars) right in the same range as the signal intensities of single telomeres signals in TIF (red bars). Given that these telomeric doubles strongly resemble telomeric doublets found on Ras and BRafV600E induced-fragile metaphase telomeres (see Figure 4, S8A), we suggest that this is additional evidence for fragile telomeres in human cancer precursor lesions. These additional data therefore strongly support our conclusions that telomere dysfunction in cells of cancer precursor lesions is a result of oncogene driven telomeric replication stress leading to fragile telomeres, telomeric DSB formation, and consequently TDIS.

One could argue that telomeric doublets observed in TIF of cancer precursor lesions are due to clustering of multiple (dysfunctional) telomeres within a single DDR focus. Evidence for this occurring in cultured human cells has recently been published by the Reddel lab (Kaul et al., 2011 EMBO Reports **13** p52). Clustering of telomeres, however, generates combined telomere signal intensities that almost always are greater than (average) telomere signal intensities of non-TIF telomeres (Kaul et al., 2011 EMBO Reports **13** p52). Telomeric doublets in TIF of cancer precursor lesions are of similar intensities compared to other telomeres in the same cell making it much more likely that they are fragile telomeric structures.



Similarly, one could argue that telomeric doublets in TIF of cancer precursor lesions are two critically short telomeres that cluster in one DDR focus. Since the software to analyze telomere signal intensities frequently could not discriminate between the two foci, we divided the combined signal intensities by a factor of two (open dashed bars, see Figure on left, hatched bars). This generated a subgroup of ultra-short telomeres, significantly shorter than the single telomeres in TIF. While we cannot rule out the possibility that ultra-short dysfunctional telomeres cluster while longer dysfunctional telomeres do not, we believe that this possibility is less likely given that there is no published evidence supporting this model.

We have also modified the text in our conclusions accordingly (in red) in order to incorporate and discuss these new data in the context of this study.

REVIEWER: The fact that hTERT overexpression protects against OIS does not necessarily mean that it is the catalytic function of telomerase (and thus telomere length) that is involved. Telomerase is an auxiliary factor of the Wnt signaling pathway and this signaling is also involved in senescence regulation. It is important to show that the effect of hTERT overexpression depends upon its catalytic activity (by overexpressing catalytically-dead alleles).

ANSWER 6) As requested by the reviewer, we have now over-expressed a catalytically inactive and dominant negative version of hTERT (DN-hTERT) and tested whether the catalytic activity of telomerase is required to destabilize RasV12 induced senescence. As shown in Supplemental Figure S9F, catalytically inactive telomerase was unable to rescue RasV12 induced cellular senescence while wt-hTERT, as in numerous independent experiments, destabilized this growth arrest. Therefore, hTERT reverse transcriptase activity is required to destabilize RasV12 induced-senescence in human fibroblasts. As explained in our response #11 and #18 our data suggest that telomerase suppresses telomere dysfunction by adding telomeric repeats to (sub)telomeric DSBs (also called telomere/chromosome healing) that were generated due to oncogene induced DNA replication stress.

REVIEWER: the observation that Ras-induced senescent cells exhibit an increase rate of telomere dysfunction with time is interesting but the underlying mechanisms are quite obscure. Therefore, this result appears preliminary and difficult to interpret.

ANSWER 7) We believe that the reviewer is referring to the line graph in Figure 5E. The y-axis in the line graph of Figure 5E depicts the ***percentage*** of 53BP1 foci that colocalize with telomeric repeats in DDR positive cells. As shown in Figure 5D, the great majority of Ras-EV cells at day 6 after Ras transduction contain 53BP1 foci and most of these cells display more than one 53BP1 focus. The line graph in Figure 5E demonstrates that at that time point only ~25% of these 53BP1 foci in a given cell are telomeric. This is also directly illustrated in the micrographs of Supplemental Figure S9A and B. Over a time of three weeks, and as many cells remain stably arrested, 53BP1 foci "disappear" (note that the disappearance of these foci occurs primarily in cells with multiple DDR foci, as shown in Figure 5D). One likely reason for the disappearance of these foci is because they are repaired by cellular DSB repair mechanisms in the growth arrested cells (of note, non telomeric DSBs are efficiently repaired in stably growth arrested cells as demonstrated in Fumagalli et al., 2012 *Nat Cell Biology*, in press, doi:10.1038/ncb2466). Another possibility is because Ras expressing cells gradually form senescence associated heterochromatin foci (SAHF), structures that have been demonstrated to restrain DDR signaling (and DDR foci) in oncogene-expressing cells (di Micco et al., 2011. *Nat Cell Biology* **13** 292). Since the ***percentage*** of 53BP1-telomere colocalizations increases during this time (and as cells remain arrested), our data demonstrate that it is primarily the non-telomeric foci that "disappear". The telomeric foci persist (see also supplemental Figure S9B). Due to the persistence of the telomeric 53BP1 foci and the disappearance of the non-telomeric 53BP1 foci, we observe an increase of the percentage of 53BP1-telomere colocalizations with time. As mentioned above, we believe that the reason for the persistence of these telomeric 53BP1 foci is because lesions in telomeric repeats are not repaired.

Since this is an important point, we have added another line graph to Supplemental Figure S9C showing data acquired only in Ras-EV expressing cells. In this graph we show that as the average number of DDR foci/cell (in DDR positive and senescent cells) decreases over time, the % colocalizations between the remaining 53BP1 foci and telomeric repeats increases. We hope that this additional graph makes it easier for the reader to understand that there is no increase in telomeric DDR foci, but rather a steady state persistence of these TIF.

In addition, we have now added new data demonstrating that oncogenic BRafV600E also causes transient non-telomeric DDR foci and persistent telomeric DDR foci in normal human fibroblasts (Supplemental Figure S10).

Minor points :

REVIEWER: in the introduction and discussion sections, it should be fair to cite Augereau et al (Blood, 2011) showing for the first time that early stages of a human malignancy (B-cell chronic leukemia) correlate with an increased rate of telomere dysfunction (measured by TIF) associated to a senescent-like phenotype (G0 arrest, p16 activation).

Regarding the capacity of a single or limited number of telomeres can trigger senescence, it would be fair to cite Hemann et al, Cell, 2001 and Abdallah et al, NCB 2009. -« ....and telomeres resemble fragile sites (Martinez et al, 2009; Sfeir et al, 2009)» it would be fair to also cite Ye et al, Cell, 2010.

ANSWER 8) We have now cited these references in the manuscript as suggested by the reviewer. However, we were unable to find relevant information in the Hemann et al paper and have therefore

not cited this study. The main conclusion of Hemann et al is that the shortest telomere undergoes end to end fusions in mTR knockout animals, however, it does not state anything about the number of dysfunctional telomeres that are required to initiate cellular senescence.

REVIEWER 2:

Suram et al. investigate telomere dysfunction induced senescence in human carcinogenesis. The authors show evidence for telomere dysfunction induced DNA damage foci (TIFs) in early stages of 3 different types of human tumors (melanoma, breast, colon). Interestingly, one of the tumor types (melanoma) shows long telomeres yet the tumors display TIFs. The authors go on and show that oncogene activation (H-RasV12) induces replication stress and replication fork stalling at the telomere eliciting a DNA damage response reminiscent to telomere dysfunction induced senescence. The authors also present data showing that telomerase expression can rescue the activation of DNA damage signals at telomeres in context of oncogene activation. The authors propose that tumor suppression by telomeres involves oncogene induced TIFs rather than the classical model proposing that telomere shortening impairs clonal expansion of telomeres.

The authors present an interesting idea and some experimental data supporting their hypothesis. However, there are some concerns that need to be addressed:

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ANSWER 9) The reviewer raises an important question, also mentioned by the other reviewers. For these reasons we have analyzed and quantified telomere signal intensities in TIF and compared these to telomere signal intensities of all other visible telomeres in cells of nevi, ductal hyperplasias of the breast, colonic adenomas, and in Ras senescent cells. We discovered that dysfunctional telomeres are, on average, of similar lengths compared to the other telomeres, based on the fluorescence signal intensities emitted by the Cy3 labeled telomeric PNA probe (Figure 1E, and supplemental Figures 5B, 5D, and 9D). Even in individual cells, it is generally not the shortest telomere that colocalizes with the DDR focus. These data therefore strongly argue against the possibility that it is only critically short telomeres (those that lack telomeric repeats) that trigger cellular senescence in these cells. We have also included data demonstrating telomeric doublets in TIF of analyzed cancer precursor lesions, structures that resemble oncogene induced fragile telomeres, further supporting our conclusions that TIF are a result of oncogene induced DNA replication fork stalling/DSB formation in telomeric repeats. Also see answer #5 for additional information on this.

REVIEWER: This concern is even more important given that studies from Taylor Jacks have shown that endogenous activation of oncogenes is not leading to induction of senescence, which appears only in response to over expression of oncogenes at high levels in cell culture (Cancer Cell 2004).

ANSWER 10) We believe that our data are not at odds with the study mentioned by the reviewer. The primary reason is that the *Cancer Cell* study expressed oncogenic Ras (oncoRas) in mouse cells, while we conduct our studies in human cells. The *Cancer Cell* study used mouse embryonic fibroblasts (MEFs), in addition to conditionally expressing oncoRas in the lung and gastrointestinal tracts of these animals. The authors demonstrate that high levels of oncoRas caused cellular senescence in MEFs by activating the p19ARF/p53 pathway (also demonstrated in several earlier studies), while lower levels of this oncogene promoted proliferation and transformation. Similarly, low levels of conditionally expressed oncoRas *in vivo* caused preneoplastic epithelial hyperplasias without any signs of cellular senescence. However, for reasons outlined below, these results should and cannot be directly compared to our (and other investigators') data, demonstrating that oncoRas causes cellular senescence in normal human somatic cells, including fibroblasts.

There are clear and quite significant differences between mouse and human cells, including their responses to oncogenes and their ability to spontaneously transform. In mouse embryonic fibroblasts (MEFs), high levels of oncogenes (Ras) generally cause cellular senescence in a p19ARF/p53 dependent manner (for example see Wei et al., 2001 *MCB* **21** 6748). This is also mentioned in the *Cancer Cell* study by Tyler Jacks. However, ARF (p14 in humans) is not upregulated in human cells in response to oncoRas, even when RasV12 is dramatically overexpressed in multiple human fibroblast strains (Wei et al., 2001 *MCB* **21** 6748). Since RasV12 does not upregulate ARF in human cells, different levels of this oncogene might not have these opposing effects that are observed in MEFs. However, this has not been addressed experimentally in humans to the best of our knowledge.

The most important differences that are relevant to our study, however, are the differences in telomere biology. Not only do mice contain much longer telomeres compared to human cells, many mouse tissues and cells also display relatively high levels of telomerase activity (for example see Forsyth and Wright 2002 *Differentiation* **69** 188). Furthermore, mouse tissues in which telomerase activity is not detectable, still express TERT mRNA suggesting that these cells remain competent to activate telomerase activity when needed. In contrast, most human tissues and cells completely lack telomerase activity and transcript, suggesting that expression of telomerase (and consequently its activity) is permanently suppressed in most human cells. Exceptions are human stem/progenitor cells and cells of the germ line where telomerase activity has been detected. Telomerase activity clearly is not detectable in cultured human fibroblasts, the cell type that we used in our study. In contrast, MEFs, the cell type used by the *Cancer Cell* study, display telomerase activity as demonstrated by several laboratories, including the lab of Ron de Pinho, a collaborator on the Tyler Jacks *Cancer Cell* study (Greenberg et al. 1998. *Oncogene*, **16 p**1723). Thus, in order to compare the data from the *Cancer Cell* study to ours, we must compare data acquired from hTERT ***positive*** human cells that express oncoRas. In fact, when we do this our data and conclusions are strikingly similar to those of the Tyler Jacks study. We demonstrate that telomerized human fibroblasts (fibroblasts expressing hTERT), while undergoing a transient initial growth arrest in response to oncoRas (likely due to the high oncoRas expression levels), proliferate at higher rates compared to normal human fibroblasts (we have now added data demonstrating that the increase proliferation rates of Ras-hTERT cells over EV-hTERT cells is statistically significant in Supplemental Figure S9E). These cells do not acquire dysfunctional telomeres and do not undergo stable cellular senescence, similar to MEFs expressing low levels of oncoRas. Furthermore, we have now additionally added data demonstrating that low levels of an oncogene (BRafV600E) also cause a stable cellular growth in normal human cells, but an unstable transient growth arrest in telomerized human cells (Supplemental Figure S10). Based on our novel data and on numerous published data demonstrating reactivation of telomerase activity during the transition from pre-malignant to malignant human cancers (also see comments below), we conclude that the reactivation of telomerase during this period suppresses TDIS and therefore contributes to oncogene driven-malignant transformation of cells at later stages during cancer development. We have added a model to our manuscript illustrating these conclusions (Figure 7).

Conversely, if we want to compare our data acquired using ***normal*** human fibroblasts expressing oncoRas, we must compare it to data acquired using mouse cells ***lacking*** telomerase activity (such as TERT or Terc knockout cells) but retaining functional DNA damage checkpoint responses. Unfortunately, oncogene induced cellular senescence, or potentially the lack thereof due to manipulation of oncogene levels, has not been analyzed in telomerase knockout MEFs that are otherwise normal, to the best of our knowledge. Of note, the absence of telomerase clearly suppresses transformation, colony growth, and tumor initiation potential of INK4a-/- and TERT-/- MEF's expressing the oncogenes c-Myc or RasV12, suggesting that, when telomerase is not expressed, dysfunctional telomeres also counteract proliferation of mouse cells expressing oncogenes (Greenberg et al., 1999. *Cell* **97** p515). We would predict that also low levels of oncoRas, levels that do not activate and upregulate p19ARF, would induce telomere dysfunction and cellular senescence (or apoptosis) in these INK4a-/- TERT-/- MEFs.

REVIEWER: How can telomerase rescue oncogene induced replication fork stalling? The classical role of telomerase is to add telomere repeats to chromosomal ends. How could that help to rescue a stalled replication fork?

ANSWER 11) We do not have evidence that telomerase (TERT) can rescue telomeric replication fork stalling. In fact, we performed our DNA combing experiments in mouse embryonic fibroblasts, cells that inherently display telomerase activity. Thus, while we cannot rule out the possibility that telomerase also suppresses stalling of telomeric replication forks, our data suggest that it cannot do so with high efficiency.

We observe four telomeric phenotypes associated with oncogene expression: 1) stalling of telomeric replication forks, 2) fragile and aberrant telomeric structures, 3) stochastic telomere attrition, and 4) telomere dysfunction of telomeres that are not necessarily short. Thus, H-RasV12 expression does not exclusively lead to dysfunction of critically short telomeres. Rather, our data suggest that Ras causes DNA lesions in telomeric repeats which are sensed by the DDR cellular machinery. Consistent with this conclusion are data from our collaborators, demonstrating that DNA breaks in telomeric repeats are irreparable (see above). How does telomerase prevent telomere dysfunction in Ras expressing cells and thereby destabilize Ras induced-senescence?

In order to gain further insights into how telomerase can suppress telomere dysfunction in oncogene expressing cells, we now include ***three*** additional experiments in the revised manuscript: we 1) characterized whether telomerase can suppress telomeric DNA replication stress as measured by the appearance of fragile telomeres 2) analyzed whether telomerase can counteract telomere attrition in response to Ras expression, and 3) tested whether the catalytic activity of telomerase is required to destabilize RasV12 induced-senescence. Our results demonstrate that despite its ability to counteract Ras induced-telomere attrition (Supplementary Figure S8D), telomerase expression did not rescue the fragile telomere phenotype induced by Ras (Supplementary Figure S8A). In addition, unlike wt hTERT, catalytically inactive hTERT did not destabilize Ras induced cellular senescence, demonstrating a requirement for catalytic activity in this process (Supplementary Figure S9F-H). Based on these data it is likely that telomerase prevents telomere dysfunction by *de novo* addition of telomeric repeats to the 3' overhang generated at the site of a replication stress induced telomeric DSB. This activity of telomerase has been well described in both yeast and mammalian cells (for example see reviews by Pennaneach et al., 2006 *Mol. Microbiol.* **59** 1357, or JP Munrnane 2010 *Cancer Res.* **70** 4255) and is called chromosome healing (at interstitial sites) or telomere healing (at telomeric DSBs). Also see answer #18.

REVIEWER: Isn't it still more plausible that the stalled forks only represent a side effect but the true senescence inducing mechanism remains telomere dysfunction induced by loss of telomere repeats, which can be rescued by telomerase activation.

ANSWER 12) Since this possibility was also raised by the other reviewers, we now quantified fluorescence signal intensities of telomeres in TIF and compared these to the fluorescence signal intensities of telomeres that did not recruit DDR factors and therefore are functional. Analysis was performed on cells in nevi, ductal hyperplasias of the breast, colonic adenomas, and Ras senescent cells. We show that telomere signal intensities in TIF are very similar to the other telomere signal intensities in the same cells, demonstrating that it is not the length of the telomere that determines whether telomeres are dysfunctional or not (Figure 1E, and Supplemental Figures 5B, 5D, and 9D). We therefore conclude that it is the formation of telomeric lesions such as DSBs that leads to cellular senescence in cells of cancer precursors. Our conclusion is consistent with data demonstrating that telomeric DSBs induced by ionizing radiation, genotoxic drugs, and site specific endonucleases are irreparable and consequently activate a persistent DDR (Fumagalli et al., 2012 *Nat Cell Biol*, in press, doi:10.1038/ncb2466). The Fumagalli et al. study also demonstrates that telomeric DSBs result in telomere dysfunction of telomeres that are not necessarily shorter compared to other telomeres in the same cells. Also see our comments to this concern from other reviewers.

REVIEWER: This concern is strengthened by the observation that tumor formation is not significantly reduced in first generation of telomerase knockout mice (see papers of DePinho, Chin and Greenberg). First generation telomerase knockout mice have sufficient telomere repeats. If oncogene induced replication fork stalling is the main mechanism of tumor suppression in telomerase negative cells, then these mice should be well protected. However, this is not the case, but telomerase knockout mice show tumor suppression only in later generations when telomeres are critically short. These data indicate that telomere shortening rather then oncogene induced telomere dysfunction is most important in tumor suppression.

ANSWER 13) While the studies mentioned by the reviewer have dramatically improved out understanding of telomere biology and their ability to either promote or suppress cancer progression, we believe that for reasons outlined below these studies cannot be ***directly*** compared to our data.

It is correct that in some studies, but certainly not all (see below), tumor incidence is similar in early (G1) generation Terc-/- mice compared to wt Terc animals, but significantly reduced in late (G5) generation Terc-/- animals. In the absence of any oncogenic stress that, based on our data, would affect the rate of telomere erosion/dysfunction (such as in INK4a-/- Terc-/- animals) this is not surprising since telomeres in G1 Terc-/- INK4a-/- animals are still very long and therefore do not pose a barrier to tumor cell proliferation (Greenberg et al., 1999 Cell **97** p515). Unfortunately, a Ras induced tumor model more relevant for comparison with our data, with low Ras expression levels in the Terc knockout mouse, has not been published to the best of our knowledge.

We are aware of studies in which oncogenes are overexpressed in the Terc-/- mouse that come to seemingly opposing conclusions. For example, in animals that are lymphoma prone due to overexpression of the Eu-Myc oncogene, wt and G1 Terc-/- animals show similar cancer growth kinetics while G5 Terc-/- animals show reduced cancer incidence and improved survival (Feldsner and Greider 2007. Cancer Cell **11** p461). In contrast, telomerase deficiency in animals that are prone to develop mammary tumors due to MMTV-neu oncogene overexpression, reduces the number of mammary tumors and increased tumor latency regardless of telomere length (Bojovich and Crowe. 2011 Mol Carcinogenesis).

We therefore currently do not have sufficient understanding of how different oncogenes affect telomere replication/function in mice in order to compare these studies to ours. Furthermore, the contributions of tumor suppression by the DDR have not been appropriately analyzed in mice. Thus it is possible that the contribution of the DDR to suppressing tumor growth in mice is significantly different compared to humans. One can also not rule out the possibility that mice, due to constitutive telomerase expression, may have lost the ability of telomerase to prevent oncogene-induced telomere dysfunction and rely on other tumor suppressive mechanisms such as those mediated by ARF.

REVIEWER: The observation that oncogene induced senescence is not stable in TERT expressing cells is interesting. It stands in contrast with previous publications from the Weinberg lab.

ANSWER 14) We believe that our data are consistent with previous studies from the laboratory of Dr. Weinberg, Dr. Sedivy, and those of many others that previously demonstrated cellular senescence in different human fibroblast strains (and hTERT immortalized fibroblasts) between days 7 and 10 following Ras transduction. We demonstrate, just as in many other studies, that the great majority of Ras transduced cells (~90%) are senescent at days 12-14 (Figure 5C). We used BrdU incorporation (or the lack thereof) as a marker of cellular senescence. We prefer this senescence marker over another commonly used marker, the senescence associated beta-Galactosidase activity. The SA-betaGal assay also labels quiescent cells (for example see (Cristofalo

2005 Exp. Gerontology ***10*** p863) and cannot be used as a reliable quantitative marker, due to high variability in staining intensities.

In addition to previous studies from the Weinberg and Sedivy labs, numerous other studies have analyzed cellular senescence in response to oncogenic Ras signaling. In human cells, analysis is almost always performed 6-14 days after Ras transduction, the time period during which cellular senescence is most stable (for example see Fig. 5a). With the exception of very few studies (see below) most publications do not illustrate or discuss cell growth curves. To the best of our knowledge, no published study illustrates a growth curve beyond day 23 -for normal human fibroblasts- or beyond day 12 –for hTERT expressing fibroblasts-(Di Micco et al. 2006. Nature ***444*** p638). Thus, our studies are not at odds with published data. Instead, ***our data provide novel and important insights into the long term stability of oncogene induced cellular senescence***. Of note, a recent study also demonstrated that H-RasV12 does not induce a stable cellular growth arrest in hTERT expressing BJ cells (Kohsaka et al., 2011. Biochem. Biophys. Res. Comm. ***410*** p878). However, this study failed to compare the results with normal BJ fibroblasts and therefore did not come to the conclusion that RasV12 induced senescence is stabilized by telomere dysfunction.

We would also like to emphasize that we have repeated these experiments a total of 14 times. We confirmed that the presence of hTERT prevents a stable cellular growth arrest in other human skin fibroblasts such as NHF (laboratory of Dr. Janine Santos UMDNJ) and HSF43 (laboratory of Dr. Harvey Ozer, UMDNJ). We also observed a similar unstable cellular growth arrest in response to oncogenic Ras in telomerized human lung fibroblasts (LF1; laboratory of Dr. John Sedivy). We characterized four H-RasV12 expression constructs (pBabe-Ras from R. Weinberg laboratory (MIT); pBabe-Ras from Dr. O. Bischoff (Institute Louis Pasteur); pBabe-Ras from Dr. F d'Adda di Fagagna (IFOM, Italy); pRRL.SIM-18-Ras (from Dr. J. Campisi; Buck Institute). All constructs caused a stable growth arrest in normal human skin fibroblasts (with few cells proliferating at a slow rate) and an unstable growth arrest in skin fibroblasts expressing hTERT. Cells expressing hTERT always continued to grow at accelerated rates (compared to control transduced cells) after a transient period of growth arrest. Finally, we now demonstrate almost identical data using the oncogene B-RafV600E. Our data demonstrate that B-RafV600E causes stable cellular senescence in normal human fibroblasts, but only a transient cellular growth arrest in telomerized human fibroblasts (Supplementary Figure S10).

Of note, as in the case with Ras induced senescence, cellular senescence induced by BRAfV600E in telomerized human fibroblasts (BJ-hTERT cells) has been demonstrated previously (Michaloglou et al. 2005 Nature **436** 720). The authors demonstrated cellular growth arrest up to day 15 after retroviral transduction. Data for subsequent days was not reported. Consistent with these studies, we also observed a seemingly stable cellular growth arrest up to day 20 following BRAFV600E expression in BJ-hTERT cells (Supplemental Figure S10). Significantly, we show that BRafV600E-hTERT cells continued to proliferate 20 days after retroviral transduction and therefore reveal novel and important insight into the long term stability of this growth arrest. Our data demonstrate that hTERT can also destabilize BRafV600E induced cellular senescence by suppressing the formation of dysfunctional telomeres.

REVIEWER: The authors should demonstrate a mechanism how telomerase expression can revert oncogene induced stress. Is it really a rescue in replication fork stalling? Or are other mechanisms involved, e.g. ROS? Wnt-signaling? Telomere dysfunction induced by shortening?

ANSWER 15) As mentioned above, we do not have evidence that hTERT rescues stalling of telomeric replication forks. We still observe telomeric replication stress in telomerase positive MEFs by DNA combing, and in telomerase positive human fibroblasts by the appearance of fragile telomeres. Because of our new experiments, also discussed in Answer #11, we can now also eliminate the possibility that Ras induced-senescence is destabilized in hTERT expressing cells as a consequence of altered Wnt-signaling. We base this on our new data demonstrating that catalytically

inactive DN-hTERT, which is equally competent in regulating Wnt signaling as wt hTERT (Park, et al., 2009 Nature 460 p66), was unable to destabilize Ras induced-senescence (Supplemental Figure S9). These new experiments also eliminate the possibility that hTERT expressing destabilized Ras-senescence by reducing levels of ROS, since DN-hTERT would have equally reduced cellular ROS levels. Furthermore, unpublished data from the d'Adda di Fagagna laboratory demonstrate that ROS are similarly elevated in Ras expressing cells regardless of telomerase status, demonstrating that hTERT does not alter levels of ROS in oncogene expressing cells. Overall, our data strongly suggest that telomerase counteracts telomere dysfunction by de novo addition of telomeric repeats to the site of a telomeric DSB.

REVIEWER #3:

Activation of cellular senescence programs in mouse models of cancer has demonstrated that dysfunctional telomere induced cellular senescence is as potent as apoptosis in terms of tumor suppression. However, convincing examples of dysfunctional telomere-induced senescence inhibiting the onset of human cancers are lacking. In this submission, Suram et al show that TDIS (telomere dysfunction- induced senescence) is indeed a tumor suppression mechanism in humans, using human cancer sample and human fibroblast expressing oncogenic Ras. They found that cancer precursor lesions of the skin, colon and breast but not frank malignancies display TDIS. The authors suggest that telomeres are sensitive to oncogene-induced replication stress, which results in telomere shortening and telomere dysfunction. Therefore,

TDIS mediated by oncogene-induced DNA replication stress is a potentially important tumor suppressing mechanism. In addition, the authors propose that increased hTERT activity abrogates Ras- induced telomeric DDR foci formation, thereby preventing oncogene induced senescence (and TDIS).

REVIEWER: Although the data is exciting and largely convincing, additional mechanistic insights will improve the manuscript. These authors have previously shown that oncogene induces replication stress promotes a DNA damage response, leading to checkpoint activation and the onset of cellular senescence. Therefore, the most interesting part of this study is understanding how TDIS occurs in precursor lesions. For example, what is the status of telomerase activity and/or hTERT expression in precursor lesion vs. malignant cancer? Could the authors correlate decreased hTERT activity in cancer precursor lesions with an increase in TDIS?

ANSWER 16) Indeed, we believe that, due to the absence of high telomerase activity in cells of cancer precursor lesions (see below), telomeres become dysfunctional as a result of DNA replication stress. Dysfunctional telomeres stabilize cellular senescence and consequently stop the progression of the cancer to more advanced stages. In contrast, the growth of cells in malignant cancers is not compromised by dysfunctional telomeres since telomerase activity, which can be detected at high levels in ~90-95% of all malignant human cancers, prevents telomere dysfunction. We have devoted an entire paragraph in the discussion to discuss this scenario. In addition, we have now added a Figure to the manuscript illustrating our conclusions in a model (Figure 7).

We have attempted to directly asses hTERT protein expression levels in analyzed tissue samples. However, the well known/notorious unspecificity and cross reactivity of many commercially anti-hTERT antibodies (for example see Wu et al. 2006 J Cell Sci 119 p2797) prevented us from testing multiple antibodies. The only antibodies that have been proven to be specific for hTERT are supplied by Rockland (Wu et al., 2006 *J Cell. Sci*. **119** p2797). We have extensively tested the Rockland antibodies using cell cultures and tissue (for example, see Supplemental Figure S9H). While immunoreactivity can be detected in hTERT expressing cultured human cells by immunofluorescence microscopy (not shown), we were unable to convincingly detect hTERT protein in malignant and benign tumor tissue. We concluded that these antibodies are unsuitable for our studies. We have additionally consulted with Dr. Alan Meeker (Johns Hopkins University), an

expert in telomere/telomerase detection in pathological specimen, who confirmed that currently no suitable antibodies are available to specifically detect hTERT protein in tissue (personal communication).

Better that analyzing hTERT protein levels is the measurement of hTERT activity using the TRAP assay. However, analyzing telomerase activity in tissue is technically challenging and extremely time consuming for the following reasons. First, measurement of telomerase activity requires freshly acquired tissue, preferably tissue that has not been frozen. Furthermore, telomerase activity must be measured in a senescent cell population that is free of surrounding normal epithelium and stem cell niches (since telomerase activity is high in these regions). Furthermore, analysis of 103 tumor specimen (the number analyzed in our study) would require years of collaborative research, yet it would only add limited novel information to our study.

The reason why it would only add little novel information is because telomerase activity in pre-malignant and malignant human tumor tissue has been characterized previously. It has been well established that telomerase activity is low or absent in premalignant lesions such as ductal hyperplasias of the breast and is high in invasive ductal carcinomas (for example see review by Heaphy and Meeker, 2011 *J. Cell. Mol. Med.* 15 p127 and references therein). Similarly, telomerase activity in normal colonic mucosa and adenomas is lower compared to adenocarcinomas (Tang et al. 1998. Cancer Res. 58 p40; and Saleh et al. 2008 *Pathology* 40 p25). In addition telomerase activity is low or absent in early melanocytic lesions such as nevi and some radial growth melanomas (Ramirez et al. 1999 Neoplasia 1 p42) as well as in cells that were directly derived from nevi and early malignant skin cancer lesions such as radial growth phase (RGP) melanomas (Soo et al. 2011. *Pigment Cell Melanoma Res.* 24 p490). Below, we have included the two key figures from the mentioned manuscripts:

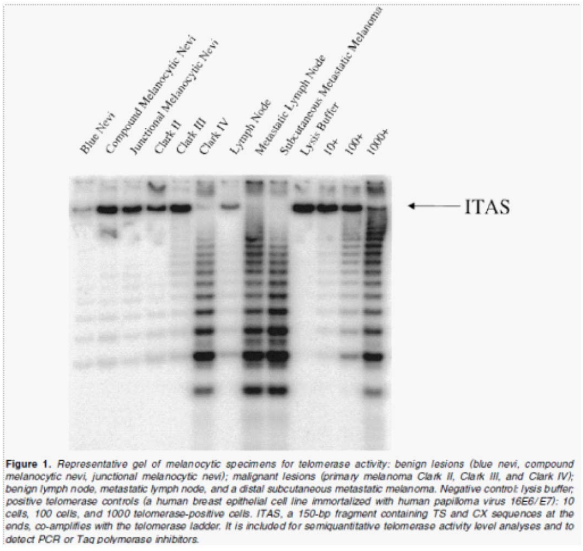
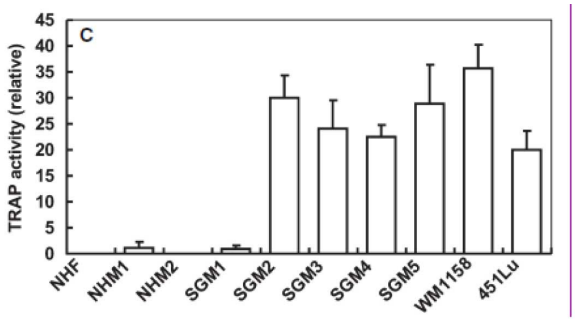


Figure 1. *Representative gel of melanocytic specimens for telomerase activity: benign lesions (blue nevi, compound melanocytic nevi, junctional melanocytic nevi); malignant lesions (primary melanoma Clark II, Clark III, and Clark IV); benign lymph node, metastatic lymph node, and a distal subcutaneous metastatic melanoma. Negative control: lysis buffer; positive telomerase controls (a human breast epithelial cell line immortalized with human papilloma virus 16E6/E7): 10 cells, 100 cells, and 1000 telomerase-positive cells. ITAS, a 150-bp fragment containing TS and CX sequences at the ends, co-amplifies with the telomerase ladder. It is included for semiquantitative telomerase activity level analyses and to detect PCR or Taq polymerase inhibitors.*

telomerase activity in human tissue samples from Ramirez et al. 1999 Neoplasia 1 p42



Cell line	Source[a]	Oncogene
SGM1	RGP	None found
SGM2	RGP	*BRAF* V600E
SGM3	RGP	*BRAF* V600E
SGM4	RGP	*BRAF* V600K
SGM5	VGP	*NRAS* Q61K
SGM6	VGP	None found

telomerase activity in cells derived from indicated lesions from Soo et al. 2011 Pigment Cell Melanoma Res. 24 p490

FIGURE: telomerase activity was measured using the TRAP assay. NHF: normal human fibroblasts; NHM: normal human melanocytes; RGP: radial growth phase melanoma; VGP: vertical growth phase melanoma)

In summary, while we could attempt to reproduce these data, we believe that the published evidence for low telomerase activity in cancer precursor lesions and high telomerase activity in over 90% of malignant human cancers is very strong and sufficient.

REVIEWER: One problem with this study is the well known observation that telomerase expression is actually elevated in advanced malignancies. If the authors are correct, increased telomerase expression should quell TDIS, thereby eliminating this driver of genomic instability.

ANSWER 17) Indeed, this is exactly our conclusion (see above). We have modified our conclusion and added a model to the manuscript (Figure 7) to better describe this point. We hope that our comments, and our model in Figure 7, make this point clearer.

REVIEWER: Other points: Considering the role of hTERT in preventing OIS, could overexpression of hTERT abrogate Ras-induced telomeric replication stress? Data from replication experiment (Figure 3) using cells with both hTERT and Ras will be helpful.

ANSWER 18) As also mentioned also in answer #1, we used TERT positive MEFs for experiments shown in Figure 3. Our data therefore suggest that telomerase cannot ***prevent*** stalling of telomeric replication forks in response to oncogenic Ras expression, just as it cannot prevent stalling of telomeric replication forks in response to TRF1 deletion (Sfeir et al., 2009 *Cell* **138** 90) or in response to aphidicolin induced DNA replication stress (Sfeir et al., 2009 *Cell* **138** 90). However, currently it is not known whether the presence of telomerase (activity) can ***reduce*** telomeric replication stress, when compared to cells lacking telomerase activity.

Another measure of telomeric replication stress is the appearance of aberrant telomeric structures in metaphase called fragile telomeres. These structures appear in telomerase positive MEFs encountering telomeric replication stress due to TRF1 ablation or aphidicolin treatment. Similarly fragile telomeres are generated telomerase positive cancer cells (HTC75) treated with aphidicolin (Sfeir et al., 2009 *Cell* **138** 90). In our previous submission we demonstrated that fragile telomeres are also generated in normal BJ fibroblasts that lack telomerase activity in response to H-RasV12 expression.

In order to determine whether telomerase can reduce DNA replication stress in telomeric repeats, we now also analyzed BJ-hTERT fibroblasts expressing RasV12 for the presence of fragile telomeres. We demonstrate that telomerase overexpression is not able to significantly reduce the generation of fragile telomeres in response to RasV12 when compared to normal BJ fibroblasts (compare Figures 4 to Supplemental Figure S8A).

In summary our data demonstrate that while telomerase ***cannot*** suppress telomeric replication stress in response to oncogenic Ras, it can prevent the formation of dysfunctional telomeres in response to RasV12 induced telomeric replication stress. We show that telomerase catalytic activity is required to suppress oncogene induced dysfunctional telomeres and to destabilize oncogene induced senescence. Based on published data demonstrating telomere elongation in telomerase positive cancer cells encountering replication stress (cells treated with aphidicolin), it is likely that telomerase prevents telomere dysfunction in these cells, by synthesizing new TTAGGG repeats to telomeres in which replication forks had stalled (Sfeir et al., 2009 *Cell* **138** 90). This is supported by our data, now added to the manuscript, that hTERT expressing cells do not shorten telomeres in response to Ras expression (Supplementary Figure S8D), while cells lacking telomerase activity do (Figure 4B-C and Supplementary Figure S8B-C). Therefore, one likely mechanism how telomerase prevents telomere dysfunction in response to DNA replication stress is that telomerase adds *de novo* telomeric repeats to the exposed 3' overhang of a telomeric DSB (or single stranded nick) close to a stalled telomeric replication fork and thereby prevents telomere attrition and dysfunction. This mechanism is not novel, it is analogous to "chromosome healing" at non telomeric DSBs and identical to "telomere healing" at telomeric DSBs and has been described previously in yeast and mammalian cells that express telomerase (for example see reviews Pennaneach et al., 2006 *Mol. Microbiol.* **59** 1357, or JP Munrnane 2010 *Cancer Res.* **70** 4255).

REVIEWER: Does BrdU incorporation rate increase in cells with Ras- hTERT at day29 compared to Ras expressing cells? Additional BrdU data from day29 group in Figure 5B will be more informative than data from day14 alone.

ANSWER 19) We added the BrdU data to Figure 5B in order to demonstrate that our results are in agreement with all previous studies that demonstrated cellular senescence (lack of BrdU incorporation) in normal human fibroblasts (regardless whether hTERT was overexpressed or not) two weeks following H-RasV12 expression. Since the presence of Senescence Associated beta Galactosidase activity (SA-bGal) is an unreliable marker of cellular senescence (for example see Cristofalo 2005 Exp. Gerontology ***10*** p863), we (as well as many other laboratories) use the lack of BrdU incorporation as a marker of cellular senescence for mammalian cells. BrdU was added to the culture medium for 48 hours to ensure that all cells capable of entering S phase would be counted as BrdU positive or as "proliferating. Conversely, cells that did not enter S phase within 48 hrs would be counted as BrdU negative or as "senescent". However, since we added BrdU for 48hrs, growth rates cannot be measured.

Based on the growth curves in Figure 5A, it is apparent that Ras-hTERT cells proliferate at significantly higher rates compared to Ras-EV cells (1PD/day vs 0.3 PD/day, respectively). Of note, as mentioned in the manuscript text, many factors influence whether Ras causes a stable or unstable cellular growth arrest in human cells. These include the age of the cells, the levels of ROS, and the activity of telomerase, among others. Since cell cultures are comprised of a highly heterogeneous population of individual cells, Ras induced DNA replication stress very likely does not affect all cells equally, forcing some cells to enter senescence immediately, while other cells, potentially with lower Ras levels, are more resistant to replication stresses exerted by Ras. Consequently, these cells will enter senescence at a later time point as indicated by the very low proliferation rates of Ras-EV cultures.

Due to the dramatic differences in growth rates between Ras-hTERT and Ras-EV cells, we are unsure why the referee suggested to measure differences using BrdU incorporation. More subtle changes, however, are the differences between proliferation rates of EV-hTERT and Ras-hTERT cells as indicated in the growth curves. These differences may be important as oncogenes often cause hyperproliferation, a feature that is also common to many cancer cells. Our novel data now suggest that dysfunctional telomeres are the primary barrier to oncogene induced hyperproliferation. In order to determine whether these differences in growth rates between Ras-hTERT and EV-hTERT cells are statistically significant, we now added BrdU to the culture medium for 8h and measured BrdU incorporation by immunohistochemistry. We now include a bar graph in Supplementary Figure S9E demonstrating that significantly more Ras-hTERT cells incorporate BrdU within 8 h compared to control hTERT only-expressing cells. Our new data therefore strengthen our conclusion that telomere dysfunction induced cellular senescence is the primary barrier to hyperproliferation observed in oncogene expressing cells.

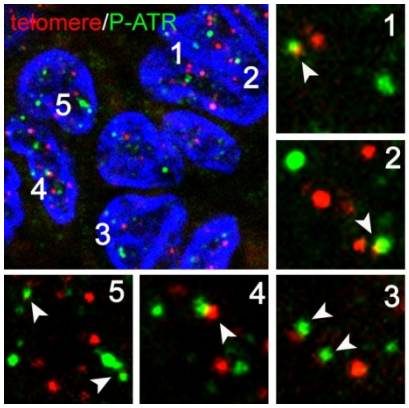
REVIEWER: Figure 5D needs to indicate statistical significance properly. Especially, a comparison between the Ras-Ev and Ras-hTERT groups at 29 days is necessary.

ANSWER 20) We thank the reviewer for noticing this omission and we have now added more statistics to this Figure. While all groups between Ras-EV and Ras-hTERT show statistically significant differences (for all days), we only show this for the day 29 groups. If we were to add bars all groups, the Figure would be illegible. Most important, in our opinion, is to demonstrate differences between the Ras-EV and EV-EV as well as Ras-EV and Ras-hTERT groups at day 29. We also included this statistical analysis for or BRafV600E experiment shown in Supplemental Figure S10C.

REVIEWER: To distinguish non-telomeric DDR and telomeric DDR in Figure 6D, co-immunostaining with telomere (PNA) and pATR will be needed.

ANSWER 21) Detecting DDR foci by immunofluorescence microscopy in tissue is not trivial. To date, the only DDR factor that has been successfully detected in tissue in discrete foci by immunofluorescence microscopy is 53BP1, to the best of our knowledge. We have tested countless additional antibodies for co-immunostaining these DDR foci, but none can visualize them as efficiently as anti 53BP1 antibodies. Two other DDR factors that also immunostain DDR foci, however significantly less efficient compared to anti 53BP1, are anti-gH2AX and anti P-ATR(S428) antibodies. We were fortunate that the anti P-ATR antibodies immunostain DDR foci that are generated by DNA replication stress, but not by other genotoxic stresses (see Supplemental Figure S12A).

In order to detect 53BP1-TIF, we additionally have to heat treat tissue in 70% formamide to 80C, conditions that significantly reduce the efficiency of antibody staining. Despite this, we can efficiently detect, image, and quantify 53BP1-TIF in tissue. We have attempted to do the same with gH2AX TIF in tissue, but due to weak fluorescence signals emitted from gH2AX foci, we were not successful in quantifying the telomere dysfunction events visualized the anti gH2AX antibodies. Based on the reviewers comment, we have now spent significant effort in attempting to visualize P-ATR TIF in human tumor tissue. While we can detect P-ATR TIF in cells of benign nevi (see Figure illustrating melanocytic cells in benign nevi; telomere: red; P-ATR: green, nuclei: blue; arrows point to P-ATR/telomere colocalizations), these events are much more difficult to image and quantify compared to 53BP1 TIF in the same lesions. Since the P-ATR foci are much smaller compared to 53BP1 foci, we have to expose the images for longer times. However, this also increases background staining, making it more difficult to distinguish non specific background staining from specific P-ATR foci. Thus, we also detect signals that likely are due to background staining. We are reluctant to include these data in the manuscript, since we currently cannot say with certainty that ***all*** green foci generated using the P-ATR antibody are specific to P-ATR.



However, the main point that we wanted to make with the micrographs shown in Figure 6 is that we can observe signs of telomeric DNA replication stress in human cancer precursor lesions. We have now added additional data demonstrating that many dysfunctional telomeres display telomeric doublets, or more diffuse staining, structures that resemble fragile telomeres. Since fragile telomeres are a hallmark of telomeric replication stress, we now have provided even more evidence that telomere dysfunction in cancer precursor lesions is a consequence of telomeric replication stress.

Furthermore, since our conclusion in the last sentence of the results section was not accurate, we have now changed the sentence to: "We show that DDR foci, which are primarily telomeric, included P-ATR(S428) (Figure 6D) supporting our conclusions that TDIS in human cancer precursor lesions was a consequence of oncogene induced-DNA replication stress."

Minor comments

REVIEWER: The figures need more details. Although the figure legends indicate details, the figures should explain the experiment as well. Immunostaining result in Figure 1D and Figure 2D has to specify for which protein is being stained.

ANSWER 22) We have added more information to Figures as requested by the referee.

REVIEWER: In figure 5E, what does the y-axis indicate? The y-axis needs to indicate whether the percentage is normalized to total cells or 53BP1 positive cells

ANSWER 23) We analyzed only DDR positive cells and have added this information to the figure legend.

2nd Editorial Decision 11 April 2012

Thank you very much for the revised study that has now been assessed by two of the original referees. As you will, one of them suggests a more focussed discussion and recommends amendment of the title. I leave theses items up to your discretion, but would be grateful for an amended word-file in case you take these suggestions up before formal acceptance.

Pending your response to this letter, the editorial office will soon be in touch with necessary paperwork and official acceptance.

Please allow me to congratulate to the study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

ref#1

The revised version adequately addressed the points raised by this referee.

ref#2

The authors have nicels addressed my concerns. I would recommend to discuss the thoughts on oncogene induced senescence and oncogene levels (Taylor JAcks paper) in the paper. Also I think the title could be changed.
"Oncogene induced telomere dysfunction activates senescence in human precursor cancer lesion"

I strongly recommend the paper for publication. It changes our way of thinking on cancer development.

Author Correspondence 12 April 2012

Thank you for allowing us to modify the title of our manuscript and to focus our discussion as suggested by one of the reviewers. All changes to the text are indicated in red coloring. We have made four minor changes:

1) The tile "Telomere Dysfunction-Induced Cellular Senescence in Human Cancer Precursor Lesions" was changed to "Oncogene-Induced Telomere Dysfunction Enforces Cellular Senescence in Human Cancer Precursor Lesions". Instead of using the term "activates", as suggested by the reviewer, we used "enforces" as it more accurately describes our findings.
2) Since our collaborator Dr. F. d'Adda di Fagagna recently received another affiliation, we added this to his address on the title page.
3) We replaced the key word "DNA damage" with "oncogene" on the title page to better reflect the new text in the title.
4) In order to focus our discussion better, we combined the information from two paragraphs in the discussion into one paragraph, and omitted the sentence "Our data therefore also demonstrate that total telomere length measurements cannot reliably predict whether telomeres in human cancer

precursor lesions are dysfunctional or not".
Since you left it to our discretion, we would prefer not to further alter our manuscript and include a discussion of how and why our data are in agreement with the Tyler Jacks study, as mentioned by reviewer #2. The primary reason is that the manuscript is already very long (56,000 characters w/o references and figure legends) and we would have to delete a significant amount of text in order to discuss this. We believe that all aspects of the discussion, as it currently stands, are critical for understanding the significance of our findings. We believe that the differences between mouse and human cells must be explained carefully and in great detail, for example in a review article, in order to accurately place all previous findings into the right context.