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Telomerase abrogates aneuploidy-induced telomere replication stress, senescence and cell depletion

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Corresponding authors: Cagatay Günes and Karl-Lenhard Rudolph, Fritz-Lipmann Institute FLI

Review timeline:

Submission date:	16 September 2014
Editorial Decision:	03 November 2014
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Editorial Decision:	27 February 2015
Revision received:	03 March 2015
Accepted:	04 March 2015

Transaction Report:

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

Editor: Thomas Schwarz-Romond

1st Editorial Decision 03 November 2014

Thank you once again for submitting your study on telomerase-dependent rescue of aneuploidy-induced cellular senescence for publication in The EMBO Journal.

The enclosed comments reveal general interest in timely presentation of these findings in The EMBO Journal. Refs#1 and #2 suggest inclusion of experiments that address the molecular basis of telomerase' rescue activity (ref#1) for instance using chemical inhibitors (ref#2). While these requests aim at solidifying your important observations, I would leave the amount and level of such further-reaching results to your discretion, based on possibly readily available results in the lab.

Please note that we only allow a single round of major revisions and that the final decision will be governed by the re-evaluation from at least one of the original referees.

Please do not hesitate to get in touch in case I can be of any assistance/to discuss feasibility, amount and timeline of necessary revisions.

Referee #1:

Aneuploidy is observed in 90% of human carcinomas, however how it arises during tumorigenesis is still a mystery. The Amon and Benezera groups have previously shown that induction of aneuploidy in primary cells is detrimental to cell survival and induces a "fitness penalty" that is somehow overcome in cancer cells. In this submission, Meena et al. suggest that telomerase activation might be responsible to overcome this fitness penalty. Telomerase expression in cells artificially induced to become aneuploid largely rescued aneuploidy induced senescence phenotypes as well as replication defects. While this correlation between telomerase expression and tolerance of aneuploidy induced phenotypes is intriguing, this paper suffers from lack of mechanistic insights into exactly what functions telomerase performs to erase the cellular defects induced by aneuploidy.
Major questions:
1. Is cellular proliferation defects/senescence phenotypes observed in aneuploidy induced cells p53 dependent?
2. How does telomerase expression abrogate telomere replication induced stress? Is single-stranded DNA production (as revealed by RPA2 localization to telomeres) at fragile telomeres reduced? How does this happen mechanistically? Is the TPP1-POT1 heterodimer, which localizes to single-stranded telomeres and interacts with and recruits telomerase to telomeres, required for this function?

Referee #2:

This manuscript by Meena et al presents an interesting set of studies pointing to new regulators aneuploidy, linking induction of aneuploidy with replication stress at telomeres, and demonstrating the capacity of telomerase activation to bypass the replication stress checkpoint in primary cells. The identification of factors enabling aneuploidy cells to survive and proliferate is emerging as an important area in cancer biology and in homeostasis of various tissues. The present studies provide valuable new insights into control of ploidy and in bypass of checkpoints limiting growth of aneuploid cells. Experiments that would strengthen the manuscript are listed below.

The manuscript consists of two main components-the identification of regulators of aneuploidy and the investigation of the relationship between telomere regulation and the aneuploidy-induced senescence checkpoint. The aneuploidy regulators are studied at the cell biologic level and validated to induce aneuploidy when knocked out, but their more specific functional roles are not defined. As a general suggestion, the authors should consider the incorporation of a set of complementary studies corroborating the key points using dihydrocytochalasin B, the cytokinesis inhibitor/actin polymerization inhibitor. This would enable examination of telomere function in aneuploidy independently of the RNAi studies

1. In Figure 1C, are the colors switched in the key?
2. It would be valuable to include RNAi rescue studies for some of the main phenotypes to control for off-target RNAi effects
3. The extent of the senescence response is not clear. Is there an acute cell cycle effect?
4. The replication stress checkpoint is nicely documented by immunofluorescence. The inclusion of immunoblots for p21 and also DNA damage checkpoint machinery would further support the data
5. The data in Figure 2c, g, k is based on pooled samples from different knockdowns. These should probably be presented individually.
6. A recent study from the Pellman group (Ganem, Cell, 2014) has identified the Hippo pathway as a key sensor of aneuploidy. It would be interesting to determine whether GJB3, RXFP1, or OSBPL3 knockdown induce Yap phosphorylation
7. Some figure concordances are missing - e.g pg 8, Supplementary Fig G-H)

Referee #3:

This study addresses an important question being asked in the field of Aneuploidy, namely "how does aneuplody contribute to DNA breaks and elicit a DNA damage response?". The authors very nicely show a potential mechanism via the induction of replication stress at telomeres after cells become aneuploid. After inducing aneuploidy via shRNA knockdowns from their related study, the authors found aneuploidy-induced senescence, accompanied by increased numerical and structural chromosomal aberrations. This was caused by a DNA damage response induced by telomere replication stress (shown by several conclusive experiments) which could be rescued by telomerase expression. Telomerase expression rescue experiment was also shown in HSPCs to conclusively show their point in vivo.

The authors show all these points conclusively with their straightforward, carefully-designed experiments and it was a pleasure to read the manuscript.

Listed below are some very minor points which will benefit the paper:

Minor Points:

1. Page 3, 2nd para:
The following Reference should be included immediately after "by increasing loss of heterozygosity": Baker et al, Cancer Cell 2009 (van Deursen group)

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3. Material and Methods should indicate Source of telomerase-negative and telomerase-positive cell lines. (ATCC, from lab, etc).

1st Revision - authors' response 01 February 2015

Referee #1:

Aneuploidy is observed in 90% of human carcinomas, however how it arises during tumorigenesis is still a mystery. The Amon and Benezera groups have previously shown that induction of aneuploidy in primary cells is detrimental to cell survival and induces a "fitness penalty" that is somehow overcome in cancer cells. In this submission, Meena et al. suggest that telomerase activation might be responsible to overcome this fitness penalty. Telomerase expression in cells artificially induced to become aneuploid largely rescued aneuploidy induced senescence phenotypes as well as replication defects. While this correlation between telomerase expression and tolerance of aneuploidy induced phenotypes is intriguing, this paper suffers from lack of mechanistic insights into exactly what functions telomerase performs to erase the cellular defects induced by aneuploidy.

Major questions:
1. Is cellular proliferation defects/senescence phenotypes observed in aneuploidy induced cells p53 dependent?

This is a good comment. Our data showed that aneuploidy induced senescence (AIS) coincides with activation of the p53/p21 pathway (Fig. 1 panels D and E; Figure S2, panels H and J). To test the functional relevance of the intactness of the p53/pRb pathways for AIS we introduced aneuploidy-inducing shRNAs into primary human IMR90-fibroblast that expressed the SV40-ST/LT antigens (impairing both p53 and Rb checkpoint function). This intervention abrogated aneuploidy induced premature growth arrest as compared to BJ/IMR90-fibroblast with intact checkpoints (revised Figure 1, panel F). Of note, the induction of aneuploidy in checkpoint deficient fibroblasts was more severe compared to checkpoint proficient fibroblasts (compare revised Fig. 1, panel G to Fig. 1A) illustrating that activation of the p53/pRb checkpoints indeed contributed to abrogate proliferation of

human cells in response to aneuploidy.

2. How does telomerase expression abrogate telomere replication induced stress? Is single-stranded DNA production (as revealed by RPA2 localization to telomeres) at fragile telomeres reduced? How does this happen mechanistically? Is the TPP1-POT1 heterodimer, which localizes to single-stranded telomeres and interacts with and recruits telomerase to telomeres, required for this function?

This is a very good question. Our data clearly indicate that telomerase expression rescues AIS. This is accompanied by reduced binding of pRPA2 at telomeres (see Figure 3, panels A *vs* B and panels F-H). Our interpretation is that telomerase promotes telomere replication as it has been shown in studies on yeast (Chang et al. 2009).
We included new experimental data and carefully discuss the possible involvement of POT1 in mediating the telomerase-dependent rescue in telomere integrity in response to aneuploidy induction (page 9 last paragraph through page 10 first paragraph of the revised manuscript):
"Studies in ciliates showed that telomerase assists the opening of telomeric G-quadruplexes (required for telomere replication) by interacting with ciliate POT1/TPP1 complex (Paeschke et al, 2008). Interestingly, POT1 knockdown by itself was one of the hits in one of our repeat screens on aneuploidy inducing shRNAs (data not shown). Using two different shRNAs against POT1 confirmed that POT1 knockdown led to an induction of aneuploidy in primary human fibroblast Supplementary Fig. S5A, B). Similar to the results on the other aneuploidy inducing shRNAs (see above), POT1 knockdown led to telomeric DNA damage and AIS (Supplementary Fig S5 A, C, E, G, I). Telomerase overexpression rescued induction of telomeric DNA damage and AIS in response to POT1 knockdown (Supplementary Fig S5 D, F, H). These data suggest that telomerase can rescue telomere replication when Pot1 level become limiting. The data do not exclude the possibility that the remaining 15-30% of POT1 expression in our shRNA experiments (Fig. S5A) contribute to the telomerase-mediated rescue in telomere integrity by mediating telomerase binding to telomeres. Interestingly, studies on Pot1b null mice revealed that telomerase haploinsufficiency severely impairs the survival of Pot1b deficient mice by impairing the maintenance of highly proliferating stem and progenitor cell compartments (He et al, 2009). These data suggest that telomerase can rescue telomere replication in a Pot1b independent manner in mice."

It would be interesting to test the functional role of POT1 for telomerase mediated telomere replication in aneuploid cells using CRISPR/CAS technology to completely knockout POT1. However, this could be detrimental for growth of human fibroblasts and may require the introduction of loxP sited to allow inducible deletion of Pot1. We would definitely be interested to analyze this question in greater detail in future studies and we thank the reviewer for this insightful comment.

Referee #2:

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Answer: We followed the referees suggestion and addressed this interesting question in the revised manuscript (page 10 last paragraph through page 11 first paragraph):
"Transient tetraploidy can be an initiating event leading to the induction of aneuploidy (Fujiwara et al., 2005; Andreassen et al., 1996). To examine whether tetraploidy would also provoke telomere replication stress and a senescence arrest as seen in AIS, primary human fibroblast were treated with the cytokinesis inhibitor dihydrocytochalasin B (DCB). In accordance with previous studies (Ganem et al, 2014) DCB treatment resulted in rapid induction of tetraploidy (Supplementary Fig S6A-D). DCB-treatment induced a rapid proliferation arrest, which however was independent of telomerase gene status and not rescued by telomerase overexpression (Supplementary Fig S6F). Moreover tetraploidy-induction by DCB-treatment did not result in an accumulation of replication stress as determined by the accumulation of pRPA2-foci (Supplementary Fig S6E). Together, these data indicate that tetraploidy induced growth arrest involves different mechanisms than AIS. It is possible that imbalances in the expression of replication factors could contribute to aneuploidy induced replication stress, which can be rescued by telomerase expression. Instead, tetraploidy induced growth arrest involves a different mechanism, which is not associated with telomere replication stress and therefore is telomerase-independent."

1. In Figure 1C, are the colors switched in the key?

Answer: The colors are corrected now

2. It would be valuable to include RNAi rescue studies for some of the main phenotypes to control for off-target RNAi effects

Answer: We appreciate the reviewer's comment. It is a good suggestion to introduce shRNA resistant cDNAs. However, overexpression studies are complicated as it is very difficult to achieve "physiological" expression levels and the overexpression as such could induce a phenotype. We decided to employ two different shRNAs for each target gene both for cell culture experiments of human cells (Figure 1A-C and Supplementary Figure 1C-D) as well as for in vivo experiments on mouse HSCs (Fig 4A-B). Together, these experiments provide strong evidence that the shRNA knockdown of target genes (as accomplished by in total 4 different shRNAs in two species) rather than off target effects induce aneuploidy.

3. The extent of the senescence response is not clear. Is there an acute cell cycle effect?
Answer: We do not observe an acute cell cycle effect in response to aneuploidy induction by candidate gene knockdown (See Fig. 1C). After introduction of aneuploidy inducing shRNAs, telomerase negative cells continue to proliferate for a few passages at normal speed but then enter premature growth arrest. Telomerase positive cells continue to proliferate at normal speed and do not enter senescence over at least 30 PD.

4. The replication stress checkpoint is nicely documented by immunofluorescence. The inclusion of immunoblots for p21 and also DNA damage checkpoint machinery would further support the data

We included p21 Western blot data (see revised Figure 1 panel D) in addition to the IF data (revised Figure 1 panel E and Suppl. Figure 2, panels H to K) as well as Western Blot results for p53 – the main component of the DNA damage checkpoint machinery (revised Figure 1 panel D). Further on, we have determined gamma-H2AX and 53BP1 levels in response to aneuploidy showing an induction of both, gamma-H2AX and 53BP1 in BJ cells, which is partially rescued by telomerase in BJ-hTERT cells (Fig2A-D and Supplementary Fig S3A-D).

5. The data in Figure 2c, g, k is based on pooled samples from different knockdowns. These should probably be presented individually.

Answer: Data are presented in the revised figure as suggested by the referee.

6. A recent study from the Pellman group (Ganem, Cell, 2014) has identified the Hippo pathway as a key sensor of aneuploidy. It would be interesting to determine whether GJB3, RXFP1, or OSBPL3 knockdown induce Yap phosphorylation

Answer: We followed the reviewer's suggestion and performed WB for phospho-YAP1. The experiments show that aneuploidy induction in primary BJ fibroblasts does not lead to a significant increase in phosphorylated YAP1 (Supplementary Fig S6G). It should be noted that Ganem et al. (Cytokinesis failure triggers hippo tumor suppressor pathway activation. Ganem NJ, Cornils H, Chiu SY, O'Rourke KP, Arnaud J, Yimlamai D, Théry M, Camargo FD, Pellman D. Cell. 2014 Aug 14;158(4):833-48.) analyzed tetraploidy induced checkpoints and showed that the Hippo pathway is a key mechanism controlling tetraploidy induced cell-cycle (G1) arrest in response to dihydrocytochalasin B (DCB) induced tetraploidy. Interestingly, our new experimental data on DCB (see response to point 1 of this reviewer) revealed that tetraploidy induced cell cycle arrest is different from aneuploidy induced cell cycle arrest. In contrast to aneuploidy-induced senescence, tetraploidy induced cell cycle arrest does not involve telomere replication stress and cannot be rescued by telomerase expression. We discuss these differences in the revised manuscript (page 11. lines 6-17).

7. Some figure concordances are missing - e.g pg 8, Supplementary Fig G-H)

Answer: The assignment of the text to the figure is now corrected as: Supplementary Fig 3G-H

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3. Material and Methods should indicate Source of telomerase-negative and telomerase-positive cell lines. (ATCC, from lab, etc).

- The list of cell lines is updated and the source of the cells is now indicated.

2nd Editorial Decision 27 February 2015

sorry, it took some time to eventually get to the details and involve the responsible people that look after data-integrity in-house.

These are the remaining items, I would kindly ask you to look into and provide using the link (for amended figure files) OR via direct E-mail (non-data related items).

- there are a few scale bars missing:

main fig 2HLM; Fig 3EJ (these would be essential)
suppl fig 2A lower panel, 2B, S5I (volountary)

- as you know, The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with this information.

-Please also provide a minimal 2 up to 4 'bullet point' synopsis, as to highlight the major novelty/advance provided by your study.

-If you were to have an integrating figure as to visualize this in the format of 550 x 150 (400max) pixel, this would facilitate featuring your study on our homepage upon formal publication.

I am very much looking forward to receive the above items that should enable rapid formal acceptance, production and publication of your paper at The EMBO Journal.

Referee #1:

The study is fine now. The authors satisfied my
major concerns and I support the paper's publication.

2nd Revision - authors' response 03 March 2015

thank you very much for the positive evaluation of our manuscript.
Attached, please find our revised version of our manuscript that includes the items that you asked for. As for the source data: these are now submitted via the online submission system. The same is the case for the ‘bullet points’ and the synopsis figure that are also attached to this email.
We hope to have fulfilled all the requirements that were raised in your email and are looking forward to a final acceptance of our manuscript.

