Subtelomeric p53 Binding Prevents Accumulation of DNA Damage at Human Telomeres

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Editor: Hartmut Vodermaier

Thank you for submitting your manuscript on subtelomeric p53 binding sites to The EMBO Journal. After some holiday-related delays, for which I apologize, we have now received input from three expert referees who have evaluated your study. As you will see from their enclosed reports, despite the interest and potential importance of the described findings, none of the referees is convinced that the presented data provide sufficiently decisive support for causal roles of subtelomeric p53 binding in telomere DNA damage protection, nor insights into the possible underlying mechanisms of such functions. We therefore unfortunately conclude that the study is in its present form not a strong candidate for publication in The EMBO Journal.
Nevertheless, we notice that especially referees 2 and 3 offer a number of constructive suggestions on how the causal connections could be strengthened and better mechanistic understanding obtained. Clearly, this would require substantial further experimental efforts and may not be trivial to achieve. Before taking a final decision on this manuscript, I would therefore like to give you an opportunity to consider and respond to the referee reports with a brief point-by-point outline on how the major issues might be addressed; and to comment on the expected feasibility of such experiments as requested by the reviewers. These tentative response (which we may in part share and discuss with referees) would be taken into account when making our final decision on this manuscript. I would therefore appreciate if you could send us such a response at your earliest convenience, ideally by early next week. Should you have any further questions in this regard, please do not hesitate to let me know.

REFEREE REPORTS:

Referee #1:

In this paper, Tutton et al. identify p53 binding sites in subtelomeric DNA. Treatment with the topoisomerase II inhibitor etoposide enhanced p53 binding to these sites. At the same time a subset of TERRA transcripts (but not the bulk of TERRA) increased. Etoposide treatment is reported to induce damage at telomeric DNA leading to telomeric DNA degradation in p53-/- but not p53+/+ cells. The underlying mechanism of the postulated telomere-protective roles of p53 remains obscure. Finally, the authors correlate histone modification changes upon etoposide treatment in the subtelomeric region with p53 status. An eRNA from 18q became also induced. However, if and how these changes might relate to the presumed telomere-protective role of p53 is unclear. Overall, the study is at a descriptive stage and the paper does not provide the mechanistic insight I expect from a publication in the EMBO Journal.

Some major concerns:

1. P53 is shown to bind to subtelomeric DNA but not to telomeric DNA. In the Southern blot of Figure 4, however, the intactness of telomeric DNA is assessed. How should p53 then protect telomeric DNA? Furthermore, gene regulatory roles of p53 elsewhere in the genome might also affect telomeric proteins and telomere stability.

2. The effects of p53 on telomeric DNA may also relate to its functions in cell cycle control upon DNA damage. DNA damage will lead to p53-dependent cell cycle arrest. Etoposide treatment in p53 mutant cells may damage telomeric DNA due to cell cycle progression.

3. The intactness of large alpha satellite DNA is also affected by etoposide. Thus, the effects may not be so telomere-specific as claimed. Indeed, gamma-H2AX staining occurred throughout the nucleus in the picture shown in Figure 4D and the brighter foci do not appear to be specifically enriched at telomeres.

Referee #2:

In their manuscript entitled "Subtelomeric p53 Binding Prevents Accumulation of DNA Damage at Human Telomeres" Tutton et al., attempt to investigate the role of direct p53 binding to telomeres. They show that following DNA damage p53 binds to subtelomeric regions and induce eRNA production as well as epigenetic changes to chromatin. Further, they show that p53 suppresses the
formation of H2AX association to telomeres and telomere DNA degradation. They propose, but do not address, that p53 binding enhances local DNA repair. And that this is related to p53 tumor suppressor function.

In general, this manuscript presents an interesting idea that relates to a very important gene. The experiments are technically well performed. Thus, the manuscript can have substantial impact and definitely can be of interest to the readership of EMBOJ. Having said that, there are at least 5 major general issues that to my opinion must be addressed before publication would be possible.

1. The authors claim that p53 binding locally protects cis-located telomeres from degradation following DNA damage. Although p53 seems indeed to bind these regions mostly through non-canonical sites, and indeed in p53 wt cells telomeres are less degraded following DNA damage, still the connection remains circumstantial. To demonstrate a direct effect of the p53 binding on the phenotypes (including epigenetic marks), the authors need to mutate one such p53 binding site using CRISPR technology, demonstrate lack of binding, and show that only the cis-located telomere is affected. This simple experiment will disconnect any doubt concerning indirect effects of p53, cell cycle etc.

2. The authors need to analyze data by GROSeq, or a comparable technology, whether the subtelomeric regions induce eRNAs following DNA damage, and whether this induction comes from the p53 binding sites, as they would predict. A typical p53-induced eRNAs effect is bidirectional originating from the near surrounding of the p53 binding site.

3. Figure 4 is to some extent problematic. The reasons are because the levels of H2AX are much higher in the p53−/− cells and therefore it is hard to know if this effect is because of the local lack of p53 binding or just general lack of p53. Second, the difference following normalization (C) is very minor, though significant. The authors need to accompany this result with cell cycle and apoptosis assays. This will also increase the connection with tumor suppressor function of p53.

4. The vast majority of the work was done using one cell line HCT-116. The authors need to demonstrate the same effects using at least another cell type, most preferably human primary cells such as fibroblasts.

5. How exactly p53 binding (local) protects the telomeres from degradation? This is not clear at all. The authors need to come with an experimental explanation; otherwise it remains more-or-less hand waving.

Referee #3:

In this manuscript, Tutton et al. identified non-canonical p53 binding sites within human subtelomeres. The authors show that in response to stress p53 binds within subtelomeres inducing the expression of a subtelomeric transcript (PARD6G) and some TERRA transcripts (13q and 2q). In addition, they investigated the functional role of p53 accumulation at telomeres in response to stress. They find that p53−/− cells in response to DNA damage agents show a greater accumulation of DNA damage foci at telomeres and a greater degree of telomeric DNA degradation. In addition, they show that p53 deficient cells in response to DNA damage agents show decreased accumulation of histone marks associated with active promoters (H3K9ac). The authors propose that p53 in response to DNA damage binds to subtelomeric regions and promotes chromosome ends protection through a direct impact on the local chromatin structure.

The demonstration of a role for p53 in the telomere protection is of interest to the DNA repair field and the telomere field. However, in addition to some experimental concerns (see below), I think that in its current form this manuscript might not have sufficient mechanistic insight into the role of p53
in this context for a high impact journal like Embo.

1- All the functional assay performed in this study replay on a single cell line (HCT116 p53 / ). The authors should use other cells types (e.g. p53 MEFs) or use complementation experiments. This would allow the authors to exclude that additional alternations are contributing to the loss of telomere protection observed in the HCT116 p53 / cell line.

2- Is the transactivation domain of p53 required for the observed phenotypes?

3- The authors propose that different p53-interacting proteins might be required for the p53-dependent protection of telomeres (e.g. CBP/p300, ATM, p53BP1). When possible the authors should and establish whether any of these factors is required for either p53-mediated inhibition of DNA damage accumulation at telomeres and telomeric DNA degradation.

4- The authors observed a drastic degradation of telomeric DNA observed in p53 -/- cells upon etoposide treatment. It was unclear to me how long after the etoposide treatment cells were harvested for telomere length analysis. What is the mechanism for this rapid telomere shortening? Is this process similar to what described previously by the de Lange laboratory as "t-loop HR events" (Wang et al., Cell 2004)?

Additional author correspondence 04 February 2015

Thank you for serving as editor for our manuscript, and for the opportunity to respond to reviewers' comments. We believe that we can provide appropriate follow up experiments to address all of the reviewers’ major concerns.

We provide a point-by-point response to each comment and how we propose to address the concern, either with new experimental data, or by modification of the existing text.

While we can not guarantee that each of the proposed experimental approaches will proof technically successful, we believe that most will be achievable in a relatively short period of time. We hope you find the plan reasonable, and leave open the option of submitting a revised manuscript to EMBO J.

Point-by-point-response:
General Comments:

Mechanistic Insight: The overwhelming concern is that our manuscript fails to demonstrate the p53 binding to the subtelomeres is directly and mechanistically involved in providing telomere DNA protection. In response, we propose several additional experiments to address these concerns, several of which are underway. However, I would also like to emphasize that the existing findings are novel, significant, and mechanistic. It is also important to highlight that this paper provides new genome discovery research regarding the binding of p53 to human and mouse subtelomeres. Validation of this observation occupies important real-estate in this manuscript. Using both ChIP-Seq experimental and publically available data sets, we find p53 binding sites in a cohort of subtelomeres. We show that the site closest to the telomere repeat track in chromosome 18q is sufficient for p53 responsiveness in reconstituted plasmid based reporter assays. We also show that telomeres have p53-dependent sensitivity and p53-dependent cell viability in response DNA damaging agent etoposide.

With respect to mechanism, we compare p53 positive and negative cell line (a well-established pair in p53 research) that have very different chromatin responses at the subtelomeric regions surrounding the p53 binding sites. The chromatin response, we propose, is the biochemical mechanism through which p53 binding provides protection to the subtelomere and other regions of the genome. We show that p53 induces histone H3K27ac and H3K9ac, and suppresses the accumulation of γH2AX throughout the region between the p53 site and the telomere repeat. We show this for two different subtelomeres, which show nearly identical pattern. We also show that this correlates with the induction of a non-coding RNA species at the p53 binding site. This is consistent with several new reports on how p53 responds to DNA damage and functions to generate enhancer-RNA (eRNA) that facilitate transcription activation at a distance (Melo et al., 2013. Mol. Cell 49:524). We show that p53 binding induces neighboring transcripts, one of which is TERRA and the other is the PARDG6 gene (for chromosome 18q).

Mechanistic explanations can be infinitely regressive and reductive. We believe we provide important and novel mechanistic insight into how p53 may provide genome protection. Our findings suggest that p53 binds to many sites that are intergenic, consistent with recent reports on p53 binding genome-wide (Kenzelmann Broz et al, 2013. GenesDev 27:1016). Among these sites are those found at numerous subtelomeres. The failure of p53 to function (null cells) leads to an increase in telomere DNA damage (TIFs) and rapid, catastrophic loss of telomere DNA (Southern blot signal loss). The mechanism is partly accounted for by changes in chromatin surrounding p53 binding sites. How the increase in histone acetylation leads to a protection against DNA damage has been a challenging question, but may be similar to how histone acetylation leads to an increase in transcription. In both cases, the increase accessibility to DNA is likely to facilitate transcription and repair. The precise mechanism is likely to be complex. Even demonstrating a new association with 53BP1 or BRCA1 would
not necessarily explain all of the aspects of how increase acetylation drives preferential DNA repair. We believe that these questions are beyond the scope of this first report.

However, we do agree that some additional validation and mechanistic insight can be provided by new experiments. These are outlined in more detail in the specific responses to the reviewers’ comments.

Response to Reviewers’ Comments:

Referee #1
However, if and how these changes might relate to the presumed telomere-protective role of p53 is unclear. Overall, the study is at a descriptive stage and the paper does not provide the mechanistic insight I expect from a publication in the EMBO Journal.

Response: We thank the reviewer for the supportive comments, and agree that additional mechanistic studies would enhance these findings.

Some major concerns:

1. P53 is shown to bind to subtelomeric DNA but not to telomeric DNA. In the Southern blot of Figure 4, however, the intactness of telomeric DNA is assessed. How should p53 then protect telomeric DNA? Furthermore, gene regulatory roles of p53 elsewhere in the genome might also affect telomeric proteins and telomere stability.

Response: Our working model is that p53 binding to the subtelomere has a direct impact on the local chromatin which can alter neighboring telomeric chromatin and DNA structure. We show that histone acetylation can increase at p53 sites, and extend into the telomere-repeat junction. H2AX signal increases in p53 null cells at positions ~150 bp from the telomere repeat tract at telomeres with p53 binding sites that we have studies (18q and 13q). The spreading of γH2AX suggests that DNA damage is occurring, and it is likely to be occurring at telomere repeat DNA, as is supported by the Southern blot.

We do not exclude additional contributions from p53 target genes, like p21, that would indirectly affect telomere repeat stability. However, it is hard to argue that p53 is not also having a direct effect on the local chromatin and transcription of the regions immediately adjacent to the telomere repeat tracts.

Experimental Approach: To demonstrate that p53 binding to subtelomeres has a direct effect on subtelomeric DNA in response to DNA damage, we propose the following two experiments.

1) We will measure changes in telomere repeat DNA directly using dot blotting methods specific for telomere repeat. We will test whether γH2AX is enriched at
telomere repeats, and more importantly, whether shelterin proteins (TRF2, TRF1) are reduced at telomere repeat DNA in p53 null cells after DNA damage treatment.

b) We will test whether CRISPR-deletion of p53 binding site at 18q has an effect on local chromatin in the subtelomere using ChIP-qPCR. We will attempt to measure 18q telomere repeat length changes by a modified STELA assay for this chromosome.

2. The effects of p53 on telomeric DNA may also relate to its functions in cell cycle control upon DNA damage. DNA damage will lead to p53-dependent cell cycle arrest. Etoposide-treatment in p53 mutant cells may damage telomeric DNA due to cell cycle progression.

Response: One experiment that we have used to address the potential indirect effects of p53 on cell cycle arrest is to follow the time course of chromatin changes at the subtelomeres. We find that the γH2AX and histone modifications occur within the first 3 hrs, suggesting that indirect cell cycle arrest and protein-synthesis of new response proteins are unlikely to account for these relatively early responses. Also, the direct measure of changes at the telomeres that have p53 binding sites are concordant with a direct role of p53 at these telomeres.

Experimental Approach:
a) We can determine whether chromatin changes occur at telomeres lacking p53 (e.g chromosome 17p).

b) We will determine whether these effects are p53 binding site dependent using CRISPR knock out of p53 binding site at chromosome 18q.

3. The intactness of large alpha satellite DNA is also affected by etoposide. Thus, the effects may not be so telomere-specific as claimed. Indeed, gamma-H2AX staining occurred throughout the nucleus in the picture shown in Figure 4D and the brighter foci do not appear to be specifically enriched at telomeres.

Response: We do not claim that the effects are telomere-specific. We discuss the alpha satellite result and, although we have not investigated a direct role of p53 binding near this repeat region, would suggest that p53 or related factors (e.g. p63 or p73) may provide some direct protection to these repetitive DNA regions similar to how p53 protects telomeres through a direct interaction with subtelomeric DNA regions.

Referee #2
In their manuscript entitled "Subtelomeric p53 Binding Prevents Accumulation of DNA Damage at Human Telomeres" Tutton et al., attempt to investigate the role of direct p53 binding to telomeres. They show that following DNA damage p53 binds to subtelomeric regions and induce eRNA production as well as epigenetic changes to chromatin. Further, they show that p53 suppresses the formation of γH2AX association to telomeres and telomere DNA degradation. They propose, but do not address, that p53 binding enhances local DNA repair. And that this is related to p53 tumor suppressor function.

In general, this manuscript presents an interesting idea that relates to a very important gene. The experiments are technically well performed. Thus, the manuscript can have substantial impact and definitely can be of interest to the readership of EMBOJ. Having said that, there are at least 5 major general issues that to my opinion must be addressed before publication would be possible.

Response: We thank the reviewer for the supportive comments.

1. The authors claim that p53 binding locally protects cis-located telomeres from degradation following DNA damage. Although p53 seems indeed to bind these regions mostly through non-canonical sites, and indeed in p53 wt cells telomeres are less degraded following DNA damage, still the connection remains circumstantial. To demonstrate a direct effect of the p53 binding on the phenotypes (including epigenetic marks), the authors need to mutate one such p53 binding site using CRISPR technology, demonstrate lack of binding, and show that only the cis-located telomere is affected. This simple experiment will disconnect any doubt concerning indirect effects of p53, cell cycle etc.

Response: We agree, and have in progress CRISPR lines lacking p53 binding sites at 18q. We will test these mutations for their response to DNA damage by measuring γH2AX and H3K27ac formation similar to experiments shown for p53 positive and negative cell lines.

2. The authors need to analyze data by GROSeq, or a comparable technology, whether the subtelomeric regions induce eRNAs following DNA damage, and whether this induction comes from the p53 binding sites, as they would predict. A typical p53-induced eRNAs effect is bidirectional originating from the near surrounding of the p53 binding site.

Response: First- the observed increase in RNA species at the p53 sites is reported since it reflects the change in the chromatin state of the subtelomeric region. The precise characterization of this non-coding RNA is beyond the scope and focus of this manuscript. We will modify the text such that we only compare the observed RT-PCR product to what others have reported to be eRNAs at p53 binding sites in known enhancers. For this report, we feel that this level of characterization should be sufficient. However, if the reviewer insists on the "run
on" experiment to demonstrate new RNA synthesis, we can provide this additional work.

3. Figure 4 is to some extent problematic. The reasons are because the levels of γH2AX are much higher in the p53-/- cells and therefore it is hard to know if this effect is because of the local lack of p53 binding or just general lack of p53. Second, the difference following normalization (C) is very minor, though significant. The authors need to accompany this result with cell cycle and apoptosis assays. This will also increase the connection with tumor suppressor function of p53.

Response: First, the p53 dependent suppression of global γH2AX has not been reported previously. We feel that this observation strongly supports our hypothesis that p53 functions globally to reduce γH2AX formation at very early stages of the DNA Damage Response.

Secondly, since there is always some level of DNA damage, the measure of clonogenicity in the absence of etoposide should show some preferential growth for p53 positive cells. We subtracted that background, although the background is most likely an important reflection of p53 function in normal cell growth and DNA replication (e.g. in the absence of etoposide).

Experimental Approach: We can add data analyzing the effect of p53 on cell cycle and apoptosis. However, it is mostly well established that p53 functions in these two cellular responses, and there is no way to attribute either of these cell responses to telomeres, short of some very complex genetic manipulations. Others have assayed the requirement for p53 in these different global functions, and it is not clear that our repeating these experiments will address the telomere specific role of p53.

4. The vast majority of the work was done using one cell line HCT-116. The authors need to demonstrate the same effects using at least another cell type, most preferably human primary cells such as fibroblasts.

Response: We show that very similar results with respect to TERRA induction can be observed in another cell type, namely the small lung cell carcinoma cell line H1299 with inducible p53 gene. These experiments were designed to compare matched cell lines that are p53 positive or negative.

Experimental Approach: We will try to deplete p53 from primary human fibroblasts using siRNA depletion to measure potential changes in telomere stability and chromatin structure in response to DNA damage.

5. How exactly p53 binding (local) protects the telomeres from degradation? This
is not clear at all. The authors need to come with an experimental explanation; otherwise it remains more-or-less hand waving.

Response: Our model (Fig. 6E) proposes that p53 binding directly alters the local chromatin by inducing histone H3K27 and H3K9 acetylation. This can be mechanistically explained by the well-established function of p53 in transcription activation through its ability to recruit several histone acetyltransferases (e.g. CBP/p300, P/CAF). How this correlates with suppression of γH2AX formation and telomere repeat DNA protection remains unknown, at this point. We speculate that chromatin acetylation increases the accessibility of telomeric DNA to repair enzymes (or resolvases) that prevent the rapid loss of telomere repeat DNA. We tested whether 53BP1 or BRCA1 were selectively recruited to subtelomeric DNA in a p53-dependent manner, but were unsuccessful in demonstrating a stable interaction with subtelomeric DNA by ChIP assay.

Experimental approach: We will test whether p53 affects shelterin proteins (TRF1 and TRF2) interaction with the telomere repeat DNA in response to DNA damage using ChIP and dot blotting. This will provide evidence that p53 is important for telomere remodeling in response to DNA damage. We will also test whether CRISPR deleted p53 binding sites have any effect on the chromatin state in response to DNA damage.

Referee #3

(Report for Author)

In this manuscript, Tutton et al. identified non-canonical p53 binding sites within human subtelomeres. The authors show that in response to stress p53 binds within subtelomeres inducing the expression of a subtelomeric transcript (PARD6G) and some TERRA transcripts (13q and 2q). In addition, they investigated the functional role of p53 accumulation at telomeres in response to stress. They find that p53/- cells in response to DNA damage agents show a greater accumulation of DNA damage foci at telomeres and a greater degree of telomeric DNA degradation. In addition, they show that p53 deficient cells in response to DNA damage agents show decreased accumulation of histone marks associated with active promoters (H3K9ac). The authors propose that p53 in response to DNA damage binds to subtelomeric regions and promotes chromosome ends protection through a direct impact on the local chromatin structure.

The demonstration of a role for p53 in the telomere protection is of interest to the DNA repair field and the telomere field. However, in addition to some experimental concerns (see below), I think that in its current form this manuscript might not have sufficient mechanistic insight into the role of p53 in this context for a high impact journal like Embo.
Response: We appreciate the supportive comments. While we provide some mechanism as to the changes in chromatin, and suppression of γH2AX, we feel that the reviewer does not credit the observation of new epigenomic data as significant value to the field. In particular, a direct role of p53 at subtelomeres and altering subtelomeric chromatin in response to DNA damage is highly significant and novel for telomeres and for p53 biology. Nevertheless, we propose additional mechanistic studies as detailed below.

1- All the functional assay performed in this study replay on a single cell line (HCT116 p53−/−). The authors should use other cells types (e.g. p53 MEFs) or use complementation experiments. This would allow the authors to exclude that additional alternations are contributing to the loss of telomere protection observed in the HCT116 p53−/− cell line.

Response: In addition to the HCT116 matched cell line, we show in Figure 2 and Supplemental Figure S4 similar findings using the alternative cell line H1299 small lung cell carcinoma cell line with Dox inducible p53. We show p53-dependent TERRA induction (Fig. 2) and p53 dependent histone modifications at subtelomeres (Fig. S4). We focus our studies on human telomeres and subtelomeres, which are very different than mouse.

2- Is the transactivation domain of p53 required for the observed phenotypes?

Response: We will attempt to investigate whether a transactivation domain defective p53 behaves like p53 wt or null. We predict that it will not induce histone acetylation at the subtelomeres. It will be interesting to determine if the p53 transactivation domain mutant fails to suppress γH2AX formation. However, we do not have an inducible cell line p53 with transactivation domain mutant, and although we will try to use transient transfection, we have technical concerns as to how well this recapitulates the more endogenous expression of p53.

3- The authors propose that different p53-interacting proteins might be required for the p53-dependent protection of telomeres (e.g. CBP/p300, ATM, p53BP1). When possible the authors should and establish whether any of these factors is required for either p53-mediated inhibition of DNA damage accumulation at telomeres and telomeric DNA degradation.

Response: Thank you for these suggestions. These are important, but complicated and challenging experiments to execute and interpret. Our working model is that p53 recruits histone acetyltransferases (presumably p300/CBP) to increase local histone acetylation which suppresses DNA damage either by recruiting repair machinery and/or limiting the spread of γH2AX. To test the role of CBP/p300 we will attempt to deplete these proteins by si/shRNA and then assay for a potential increase in telomere DNA damage in p53 positive cells. This will address whether CBP/p300 pathway is important for the suppression of DNA
damage at subtelomeres and telomeres.

4- The authors observed a drastic degradation of telomeric DNA observed in p53 -/- cells upon etoposide treatment. It was unclear to me how long after the etoposide treatment cells were harvested for telomere length analysis. What is the mechanism for this rapid telomere shortening? Is this process similar to what described previously by the de Lange laboratory as "t-loop HR events" (Wang et al., Cell 2004)?

Response: We thank the reviewer for this suggestion. We find that telomeric DNA begins to degrade at 12 hrs, and is severely lost by 24 hrs. It is possible that p53 induced histone acetylation and TERRA transcription may facilitate t-loop resolution in response to DNA damage, and that failure to complete this efficiently in p53 null cells may result in rapid telomere loss. This may be similar to what Wang et al. observed with over-expression of TRF2ΔB.

Experimental Approach: We will test for the formation of t-circles accumulating in p53 wt and null cells treated with etoposide using 2D gels and rolling circle amplification assays.

Summary of Experiments for Revision:

Major Emphasis:

1. Test the effects of CRISPR mutation of p53 binding site in human subtelomere 18q. We will test the effect of p53 site mutation on γH2AX formation, histone acetylation, and RNA expression (18qTERRA, PARD6G, 18q e-like RNA) in response to DNA damage.

2. Test the effect of p53 on shelterins (TRF1, TRF2), telomeric chromatin (H3K27Ac, H3K9Ac, γH2AX), using ChIP assays with dot blotting of telomere repeats. We will also test the effect of p53 on t-circle formation.

3. We will test the effect of a transcription activation domain mutant of 53 on subtelomeric chromatin (H3K9Ac, H3K27Ac, γH2AX) and transcription (TERRA, eRNA, PARD6G) in response to DNA damage.

Minor Emphasis:

4. We will determine if p53 null HCT116 cells have an increase in apoptosis and/or cell cycle arrest in response to etoposide treatment (Fig. 4).
5. We will determine whether non-coding RNA (putative eRNA) expression increases in response to DNA damaging agents using a ethynyl-uridine pulse and RT-PCR analysis of specific genes.

6. Determine if siRNA/shRNA silencing of p300/CBP blocks the function of p53-mediated suppression of DNA damage in response to etoposide treatment. We will focus on DNA damage at telomeres and subtelomeres.
Thank you for sending your responses to the referee reports on your recent submission. I have now looked through them and discussed them further with my colleagues.

I realize that the experiments you propose would potentially be very helpful to establish a causal role of subtelomeric p53 binding in local damage protection, and may also provide somewhat further understanding of the underlying mechanisms. I would therefore be willing to offer you an opportunity to submit a revised manuscript for our further consideration. Given that it is currently difficult to predict the feasibility, success and outcome of all the outlined experiments, I hope you nevertheless understand that I am at the present stage not able to make strong commitments regarding the eventual acceptance of the study; all further consideration will clearly depend on the conclusiveness of the revision experiments and on how much further insight they provide in the end. In this regard, I should mention that we have a sister journal, EMBO reports, that focuses on intriguing novel observations but places less emphasis on detailed mechanistic understanding, so depending on the outcome of your revisions, this could be considered as an alternative option and you may indicate your interest in this possibility at the time of resubmission.

To just briefly answer to your general comments on balancing interesting novel observations with perceived mechanistic insights: I fully appreciate your points, and realize that pressing for ever more detailed mechanistic understanding is often not all that reasonable nor valuable. Nevertheless, in the present case I also cannot simply overlook the fact that three expert reviewers unanimously find the currently provided insights too limited for an EMBO Journal article, and that they want to at least have the underlying causality decisively established. In this respect, although I have not gone back to referee 2 at this stage, I feel that the experiments suggested in their second major point might go along way to making the study overall more compelling.

Please do not hesitate to contact me anytime should you have updates on your revision experiments, follow-up questions regarding further consideration of this study, or in case you should require an extension beyond our regular three-months revision period.

Thank you again for the opportunity to consider this work for publication, and I look forward to hearing from you in due time.
Summary Response to Editorial Comments

We have added substantial new experimental and meta-data analyses to this manuscript to support the overall novel genomic structural description as well as the mechanistic significance of p53 binding sites in human subtelomeres. Specific changes include:

1) We have included new data, as requested by reviewer 2, showing the cell cycle profile of p53+/+ compared to p53-/- cells in response to etoposide treatment (new Fig 4B). As expected, we find that p53+/+ cells have a very different cell cycle arrest response to DNA damage than p53-/- cells.

2) We provide a new experiment examining the telomeric DNA structure in p53+/+ and p53-/- cells in response to DNA damage by two-dimensional agarose gel electrophoresis and Southern blot (new Fig. 4H). We find changes in DNA structures that are likely recombination-replication intermediates (X-spikes and single stranded DNA), as well as loss of overall telomeric DNA signal. We do not see an increase in telomere circles, suggesting that the rapid loss of telomere DNA is due to exonucleolytic attack, and not through t-circle generation (response to review 3).

3) We have included new experiments showing the effect p53 on TRF1 and TRF2 binding to telomere repeat and subtelomeric DNA (Fig 5C-E). We provide dot-blot analysis of telomere G-rich and C-rich strands, and control Alu repeats for ChIP-assays treated with or without etoposide in p53 positive and negative matched cell lines. We find that etoposide treatment leads to a small, but significant increased occupancy in TRF1 and TRF2 binding at telomere repeats. TRF2 is also shown to increase occupancy at subtelomeric DNA most proximal to the telomere repeat. This occurs in response to DNA damage (etoposide treatment) and is dependent on p53. The results suggest that p53 alters the telomere DNA and/or telomeric chromatin to allow increase TRF1 and TRF2 binding, as well as to increase its interactivity with subtelomere. The interactivity with subtelomeric DNA is interpreted as an increase in DNA fold-back interaction between telomere repeat DNA and subtelomeric DNA (see new model Fig 8).

4) We provide new meta data analysis of recently published GRO-SEQ for MCF7 cells treated with p53 activator Nutlin-3a (Fig 6E-F, S7). We also integrate this data with ChIP-Seq data sets for untreated MCF7 to show histone modifications associated with active transcription and enhancer elements (H3K27ac, H3Kme1, H3Kme4) at p53 binding and regulated subtelomeric sites. These findings strongly support a model (shown in Fig 6F) that subtelomeric p53 binding sites are enhancer-like elements that stimulate transcripts within 10-50 kb, including divergently transcribed subtelomeric
transcripts and TERRA.

5) (New Fig. 7 and S8). We provide new experiment showing the effect of CRISPR deletion of p53 binding site in the 18q subtelomere. We find that deletion of subtelomeric p53 leads to a loss of p53-responsive activation of subtelomeric transcripts, including PARDG6 located ~10 kb distance, and local eRNA-like species. We also find that p53-site deletion leads to elevated local γH2AX formation in response to DNA damage consistent with the model that p53 binding to subtelomeres function as transcriptional enhancers and protect DNA from accumulating excessive or unrepairable DNA damage (model Fig. 8)

We have modified the text to accommodate these new experiments, but our overall conclusion and Discussion has not changed substantially.

Response to Referee Comments:

Referee #1

(Report for Author)

In this paper, Tutton et al. identify p53 binding sites in subtelomeric DNA. Treatment with the topoisomerase II inhibitor etoposide enhanced p53 binding to these sites. At the same time a subset of TERRA transcripts (but not the bulk of TERRA) increased. Etoposide treatment is reported to induce damage at telomeric DNA leading to telomeric DNA degradation in p53−/− but not p53+/+ cells. The underlying mechanism of the postulated telomere-protective roles of p53 remains obscure. Finally, the authors correlate histone modification changes upon etoposide treatment in the subtelomeric region with p53 status. An eRNA from 18q became also induced. However, if and how these changes might relate to the presumed telomere-protective role of p53 is unclear. Overall, the study is at a descriptive stage and the paper does not provide the mechanistic insight I expect from a publication in the EMBO Journal.

Some major concerns:

1. P53 is shown to bind to subtelomeric DNA but not to telomeric DNA. In the Southern blot of Figure 4, however, the intactness of telomeric DNA is assessed. How should p53 then protect telomeric DNA? Furthermore, gene regulatory roles of p53 elsewhere in the genome might also affect telomeric proteins and telomere stability.

Response: We provide several new experiments to address this concern. We show that TTAGGG-binding proteins, TRF1 and TRF2, have increased occupancy by ChIP assay in response to DNA damage at telomere repeat, and to a greater extent at
subtelomeric DNA adjacent to telomere repeats (Fig. 5C-E). This suggests that shelterin interactions with telomere repeat and with subtelomeres are altered in response to DNA damage in a p53-dependent manner. The structural change is likely to involve alterations in transcription, chromatin, and DNA folding, as depicted in the new model shown in Fig 8. We also provide new experiment examining the telomere DNA structure by two-dimensional agarose gel (Fig. 4H). These data show that telomeric DNA structure is altered in a p53-dependent manner in response to etoposide treatment. Finally, we provide new experiments showing that CRISPR deletion of the 18q p53 binding site results in a reduction in subtelomeric transcription and accumulation of subtelomeric γH2AX in response to DNA damage (e.g. etoposide treatment). These findings strongly suggest that p53 binding at the subtelomere is playing a direct role in regulating subtelomeric transcription and DNA protection.

2. The effects of p53 on telomeric DNA may also relate to its functions in cell cycle control upon DNA damage. DNA damage will lead to p53-dependent cell cycle arrest. Etoposide-treatment in p53 mutant cells may damage telomeric DNA due to cell cycle progression.

Response: We thank the reviewer for this comment. It is always challenging to separate direct from indirect effects of genetic disruptions, especially for such pleiotropic proteins like p53. We do provide requested data showing that p53 has a dramatic effect on the cell cycle response to etoposide (Fig. 4B). This is not unexpected. However, our data showing direct binding of p53 at subtelomeres and new data showing that a CRISPR deletion of a subtelomeric p53-binding site leads to similar local subtelomeric changes as observed with global loss of p53, strongly argues that p53 has a direct effect at the subtelomeres to which it binds.

3. The intactness of large alpha satellite DNA is also affected by etoposide. Thus, the effects may not be so telomere-specific as claimed. Indeed, gamma-H2AX staining occurred throughout the nucleus in the picture shown in Figure 4D and the brighter foci do not appear to be specifically enriched at telomeres.

Response: We do not claim that p53 function is limited to subtelomeres. We have made multiple comments to indicate that p53 has multiple global functions, as it binds to numerous sites in the genome many of which are not linked to a p53-inducible gene. We focus on the previously unknown and non-canonical subtelomeric sites that have a specific effect on telomere DNA integrity. It is likely that a similar type of p53-dependent chromosome protective effect occurs at other fragile sites, like the alpha-satellite repeats in centromeric DNA.

Referee #2

(Report for Author)
In their manuscript entitled "Subtelomeric p53 Binding Prevents Accumulation of DNA Damage at Human Telomeres" Tutton et al., attempt to investigate the role of direct p53 binding to telomeres. They show that following DNA damage p53 binds to subtelomeric regions and induce eRNA production as well as epigenetic changes to chromatin. Further, they show that p53 suppresses the formation of γH2AX association to telomeres and telomere DNA degradation. They propose, but do not address, that p53 binding enhances local DNA repair. And that this is related to p53 tumor suppressor function. In general, this manuscript presents an interesting idea that relates to a very important gene. The experiments are technically well performed. Thus, the manuscript can have substantial impact and definitely can be of interest to the readership of EMBOJ. Having said that, there are at least 5 major general issues that to my opinion must be addressed before publication would be possible.

1. The authors claim that p53 binding locally protects cis-located telomeres from degradation following DNA damage. Although p53 seems indeed to bind these regions mostly through non-canonical sites, and indeed in p53 wt cells telomeres are less degraded following DNA damage, still the connection remains circumstantial. To demonstrate a direct effect of the p53 binding on the phenotypes (including epigenetic marks), the authors need to mutate one such p53 binding site using CRISPR technology, demonstrate lack of binding, and show that only the cis-located telomere is affected. This simple experiment will disconnect any doubt concerning indirect effects of p53, cell cycle etc.

Response: We have used CRISPR to delete the subtelomeric p53 binding site in 18q (new Fig 7 and S8). We show that in two different CRISPR derived cell lines, the p53 response at subtelomere 18q is compromised for both PARDG6 and for the local eRNA-like transcripts. We also show that γH2AX accumulates to a greater extent at subtelomeric regions in CRISPR cells with p53 deleted.

2. The authors need to analyze data by GROSeq, or a comparable technology, whether the subtelomeric regions induce eRNAs following DNA damage, and whether this induction comes from the p53 binding sites, as they would predict. A typical p53-induced eRNAs effect is bidirectional originating from the near surrounding of the p53 binding site.

Response: We appreciate these suggestions. We have analyzed a recent GRO-Seq data set and integrated this with ChIP-Seq data sets for enhancer-like histone modifications (Fig. 6E and F, and S7). We find strong evidence from GRO-Seq that bi-directional transcription and enhancer-like features can be observed at subtelomeric sites. Interestingly, the p53 specific sites have low levels of bi-directional transcripts, while the enhanced promoter regions have much higher levels of transcription, which can also be bidirectional. We find that these subtelomeric transcripts are induced by Nutlin-3a induction of p53, and that enhancer-like chromatin modifications (H3K27Ac, H3K4me1, and H3K4me3) are detected at the target transcripts (e.g PARDG6), rather than directly overlapping the p53 binding sites. We therefore refer to the transcripts at the p53 binding sites as eRNA-like since they do not have all of the features of
previously described enhancer RNAs.

3. **Figure 4 is to some extent problematic.** The reasons are because the levels of γH2AX are much higher in the p53-/- cells and therefore it is hard to know if this effect is because of the local lack of p53 binding or just general lack of p53. Second, the difference following normalization (C) is very minor, though significant. The authors need to accompany this result with cell cycle and apoptosis assays. This will also increase the connection with tumor suppressor function of p53.

Response: We provide new data showing the cell cycle profile of HCT116 p53-/- and p53+/+ cells in response to etoposide treatment (Fig. 4B). As expected, these cells accumulate/arrest at different stages of the cell cycle. We agree that this is important information, but we do not feel that it changes the overall conclusions of the manuscript. P53 is well-established to have global effects on S-phase cell cycle arrest in response to DNA damage. We do not suggest that subtelomeric p53 is responsible for this global effect. We only claim that p53 binding to the subtelomere provides a local response to DNA damage that can have significant effects on telomere transcription, structure, and stability in response to DNA damage stress.

4. **The vast majority of the work was done using one cell line HCT-116.** The authors need to demonstrate the same effects using at least another cell type, most preferably human primary cells such as fibroblasts.

Response: We have shown that p53 binding is induced in response to DNA damage in several different HCT-derived cell lines and in lung carcinoma H1299 cells. Meta-data analyses examines MCF7 and mouse ES cells to demonstrate p53 binding sites at human and mouse subtelomeres. We argue that it is not feasible to repeat all of the biochemical and genetic experiments in human diploid fibroblasts or mouse MEFs.

5. **How exactly p53 binding (local) protects the telomeres from degradation? This is not clear at all.** The authors need to come with an experimental explanation; otherwise it remains more-or-less hand waving.

Response: We do not know all of the mechanistic details, but we have provided several new experiments to help build a more complete model (Fig. 8). Our data strongly demonstrates that p53 binds to multiple subtelomeres in response to DNA damage. Our new data and meta-data analysis of GRO-Seq indicates that subtelomeric RNA is induced in response to p53 activation by Nutlin-3a. We also provide new data that TRF2 binding to telomeric and subtelomeric DNA is enhanced by DNA damage. Finally, we show loss of p53 binding sites lead to accumulation of γH2AX at subtelomeres. Taken together, we propose that p53-dependent binding, chromatin modification, transcription activation, and enhancer-like activities provide local DNA protection. We suggest that increase interaction with shelterins and
telomeric DNA through increase DNA folding or looping may be part of this mechanism to reduce DNA fragility (as detected by Southern blot Fig.4F-H).

Referee #3

(Report for Author)

In this manuscript, Tutton et al. identified non-canonical p53 binding sites within human subtelomeres. The authors show that in response to stress p53 binds within subtelomeres inducing the expression of a subtelomeric transcript (PARD6G) and some TERRA transcripts (13q and 2q). In addition, they investigated the functional role of p53 accumulation at telomeres in response to stress. They find that p53−/− cells in response to DNA damage agents show a greater accumulation of DNA damage foci at telomeres and a greater degree of telomeric DNA degradation. In addition, they show that p53 deficient cells in response to DNA damage agents show decreased accumulation of histone marks associated with active promoters (H3K9ac). The authors propose that p53 in response to DNA damage binds to subtelomeric regions and promotes chromosome ends protection through a direct impact on the local chromatin structure.

The demonstration of a role for p53 in the telomere protection is of interest to the DNA repair field and the telomere field. However, in addition to some experimental concerns (see below), I think that in its current form this manuscript might not have sufficient mechanistic insight into the role of p53 in this context for a high impact journal like Embo.

1- All the functional assay performed in this study replay on a single cell line (HCT116 p53−/−). The authors should use other cell types (e.g. p53 MEFs) or use complementation experiments. This would allow the authors to exclude that additional alternations are contributing to the loss of telomere protection observed in the HCT116 p53−/− cell line.

Response: (see response to review 2, comment 4). (We have shown that p53 binding is induced in response to DNA damage in several different HCT-derived cell lines and in H1299 cells. Meta-data analyses examines MCF7 and mouse ES cells to demonstrate p53 binding sites at human and mouse subtelomeres. We argue that it is not feasible to repeat all of the biochemical and genetic experiments in diploid fibroblasts).

2- Is the transactivation domain of p53 required for the observed phenotypes?

Response: This is an interesting question, but technically difficult and potentially beyond the scope of the work presented. We expect that p53 transcription activation is required for stimulation of subtelomeric transcription and local histone acetylation, but introduction of exogenous p53 is problematic as its over-expression can induce apoptosis and does not fully recapitulate the effects of endogenous p53.

3- The authors propose that different p53-interacting proteins might be required for the
p53-dependent protection of telomeres (e.g. CBP/p300, ATM, p53BP1). When possible the authors should and establish whether any of these factors is required for either p53-mediated inhibition of DNA damage accumulation at telomeres and telomeric DNA degradation.

Response: While the contribution of p300, CBP, ATM, and 53BP1 are very important, we were unable to ChIP any of these to the p53 binding sites at subtelomeres. We believe that this is due to technical limitations, and therefore can not address the reviewers important question. However, we feel that we have provided sufficient information in other directions regarding the direct role of p53 binding at subtelomeres to justify positive evaluation of our manuscript.

4- The authors observed a drastic degradation of telomeric DNA observed in p53 -/- cells upon etoposide treatment. It was unclear to me how long after the etoposide treatment cells were harvested for telomere length analysis. What is the mechanism for this rapid telomere shortening? Is this process similar to what described previously by the de Lange laboratory as "t-loop HR events" (Wang et al., Cell 2004)?

Response: We provide a time course of etoposide treatment analyzed by ChIP and RT-qPCR (Fig. 6). The Southern blot (Fig. 4) showing loss of telomere DNA is at 24 hrs post-treatment with etoposide. We provide new data (Fig. 4H) showing a two-dimensional agarose gel to investigate the potential formation of telomere circles, as was described in Wang et al, Cell 2004. However, we did not observe any significant increase in telomere circles in response to etoposide treatment, with or without p53. We did observe formation of more complex recombinational-replication structures (including X-spike and single stranded DNA), as well as the loss of telomeric signal in response to DNA damage. We conclude that the loss of telomere signal is due to exonucleolytic attack, similar to that observed when telomeres are uncapped due to loss of shelterins.
Thank you for submitting your revised manuscript for our consideration. It has now been re-reviewed by referees 2 and 3, who had originally come up with a number of constructive criticisms for improvement of the study. While referee 2 is generally satisfied with the revisions (but for one point, see below), referee 3 however retains several major criticisms.

Given the major extensions to the study, including the CRISPR deletion of a subtelomeric p53 binding site to validate underlying causalities and directness of effects, I would in this case not insist on further mechanistic exploration such as demanded in referee 3’s point 3 and in their general concerns. Further discussing referee 3’s request about the role of the p53 transactivation domain, which indeed could be very insightful, referee 2 agreed that the technical difficulties with re-expressing mutated (and - as a crucial control - wild-type) p53 would make such an experiment very difficult to conduct and/or interpret, likely requiring dedicated genetic knock-in experiments in the future.

On the other hand, there remains the point about extension to additional, ideally primary cell lines. Referee 3 reiterates the importance of this issue, and referee 2, who originally had the same concern, agreed during cross-referee commenting that this should still be addressed. While I do appreciate your argument that it may not be feasible to repeat all the biochemical and genetic experiments in human diploid fibroblasts or mouse MEFs, I would therefore still consider it essential to assess and validate at least a number of key results, such as changes in telomere stability and chromatin structure upon DNA damage, also in p53-depleted primary fibroblasts, as initially proposed in your first response letter.

In this light, I would like to return the paper to you once more for an exceptional second round of major revision, to allow you to address this remaining key issue. Should you be able to confirm key biochemical and genetic data in different cellular systems (I am happy to discuss any proposals on which experiments would be most crucial and/or feasible to recapitulate in this way), then we should be able to consider a re-revised manuscript further for eventual publication. Please understand that this will however have to be the final round of (experimental) revision, and that this remaining point will have to be addressed to our and the referees’ satisfaction at this stage. Should you have any questions in this regard, please do not hesitate to contact me.

REFEREE REPORTS

Referee #2:

the authors have answered all my request

Referee #3:

In this revised manuscript, Tutton and colleagues identified non-canonical p53 binding sites within human subtelomeres. The authors propose that p53 in response to DNA damage binds to subtelomeric regions and promotes chromosome ends protection through a
not well-defined direct impact on the local chromatin structure. The experiments are well performed and presented in a very nicely. My major concern for the previous version of this manuscript was the lack of mechanistic insight into the mechanism of action of p53 in this context. Unfortunately, the authors did not gain sufficient insights into this regard. As a result the lack of data shading light into the mechanism by which p53 protects chromosome ends remains an issue. In addition, I had a number of technical concerns that, unfortunately, the authors decided to ignore. I will, therefore, reiterate my points further highlighting the reason I think performing these experiments would be important to enhance this manuscript.

1- All the functional assay performed in this study replay on a single cell line (HCT116 p53 / ). The authors should use other cells types (e.g. p53 MEFs) or use complementation experiments. This would allow the authors to exclude that additional alternations are contributing to the loss of telomere protection observed in the HCT116 p53 / cell line. A different reviewer raised this critical point as well. The authors decided to ignore this point due to feasibility. The authors cannot avoid repeating some of the biochemical and genetic experiments in different cells lines. This is critical to exclude that additional HCT116-specific alteration are associated with the observed phenotypes.

2- Is the transactivation domain of p53 required for the observed phenotypes? This experiment would allow the authors to distinguish between a secondary effect induced by p53-responsive genes and the direct action of p53 at chromatin that is suggested by the authors. Technically this is a simple experiment that would involve complementation of p53/- cells with a transactivation deficient p53 allele. Results of these experiments could strengthen the model proposed by the authors.

3- The authors propose that different p53-interacting proteins might be required for the p53-dependent protection of telomeres (e.g. CBP/p300, ATM, p53BP1). When possible the authors should establish whether any of these factors is required for p53-mediated inhibition of DNA damage accumulation at telomeres. I have suggested these experiments since the results could provide mechanistic insight in the p53 ability to protect telomeres. The author's response is that by ChIP they could not detect these factors at chromosome ends. However, as stated by the authors, negative result by ChIP does not represent a valid reason to exclude the role of any of these factors on these processes. Down-regulation of these proteins could help the authors test their model and provide a mechanistic insight in this process.

2nd Revision - authors' response 23 October 2015
Re: EMBOJ-2014-90880R
Subtelomeric p53 Binding Prevents Accumulation of DNA Damage at Human Telomeres

Dear Paul,

Thank you for submitting your revised manuscript for our consideration. It has now been re-reviewed by referees 2 and 3, who had originally come up with a number of constructive criticisms for improvement of the study. While referee 2 is generally satisfied with the revisions (but for one point, see below), referee 3 however retains several major criticisms.

Given the major extensions to the study, including the CRISPR deletion of a subtelomeric p53 binding site to validate underlying causalities and directness of effects, I would in this case not insist on further mechanistic exploration such as demanded in referee 3’s point 3 and in their general concerns. Further discussing referee 3’s request about the role of the p53 transactivation domain, which indeed could be very insightful, referee 2 agreed that the technical difficulties with re-expressing mutated (and - as a crucial control - wild-type) p53 would make such an experiment very difficult to conduct and/or interpret, likely requiring dedicated genetic knock-in experiments in the future.

On the other hand, there remains the point about extension to additional, ideally primary cell lines. Referee 3 reiterates the importance of this issue, and referee 2, who originally had the same concern, agreed during cross-referee commenting that this should still be addressed. While I do appreciate your argument that it may not be feasible to repeat all the biochemical and genetic experiments in human diploid fibroblasts or mouse MEFs, I would therefore still consider it essential to assess and validate at least a number of key results, such as changes in telomere stability and chromatin structure upon DNA damage, also in p53-depleted primary fibroblasts, as initially proposed in your first response letter.

Response. We provide new data comparing human diploid fibroblasts with or with shRNA targeting p53 (revised Figure S9). We demonstrate a p53-dependent suppression of γH2AX globally (by Western blot), and at the 18q and 13q subtelomeres (by ChIP assay). We also find a p53-dependent enrichment of the enhancer-like RNA at the 18q subtelomeric p53 site and at PARD6 gene in response to etoposide treatment. We also show a p53-dependent increase in histone H3K27ac at the 18q subtelomere. These studies indicate that the subtelomeric chromatin modifications and p53-dependent RNA expression and activity are nearly identical between the human diploid fibroblasts and the HCT116 and H1299 cell lines. While we were not able to complete all of the assays in the limited time, we believe that this new data addresses the major concerns of the Reviewer 3, and demonstrates that p53-dependent effects on subtelomeric chromatin and transcription are essentially the same in diploid fibroblasts as in HCT116 model cell lines. We provide this new data in Supplementary Figure S9. We believe this data is more appropriate in the supplement since it does not add substantially new conceptual information, but rather validates the...
observations in a diploid fibroblasts.

It is also important to mention that a study using mouse MEFs would not be appropriate for our paper since all other experiments focus on human cells, and the human and mouse subtelomeres are extremely divergent, including the positions of p53 binding sites relative to the telomere repeat (Fig. S2).

Referee #3:

In this revised manuscript, Tutton and colleagues identified non-canonical p53 binding sites within human subtelomeres. The authors propose that p53 in response to DNA damage binds to subtelomeric regions and promotes chromosome ends protection through a not well-defined direct impact on the local chromatin structure. The experiments are well performed and presented in a very nicely. My major concern for the previous version of this manuscript was the lack of mechanistic insight into the mechanism of action of p53 in this context. Unfortunately, the authors did not gain sufficient insights into this regard. As a result the lack of data shading light into the mechanism by which p53 protects chromosome ends remains an issue. In addition, I had a number of technical concerns that, unfortunately, the authors decided to ignore. I will, therefore, reiterate my points further highlighting the reason I think performing these experiments would be important to enhance this manuscript.

Response: We apologize for the appearance of ignoring these important concerns. We agree that these are important and significant, and have made every effort to address these many concerns experimentally in the time allowed (90days) for revisions. In cases where we could not address by direct experiment, we provide text to discuss the limitations of the study. We hope the reviewer appreciates that not all of the proposed experiments are technically achievable with statistically robust results in the time frames allowed for revision. We do provide additional data (Fig. S9) that we feel addresses a major concern raised in point 1.

1- All the functional assay performed in this study replay on a single cell line (HCT116 p53−/−). The authors should use other cells types (e.g. p53 MEFs) or use complementation experiments. This would allow the authors to exclude that additional alternations are contributing to the loss of telomere protection observed in the HCT116 p53−/− cell line. A different reviewer raised this critical point as well. The authors decided to ignore this point due to feasibility. The authors cannot avoid repeating some of the biochemical and genetic experiments in different cells lines. This is critical to exclude that additional HCT116-specific alteration are associated with the observed phenotypes.

Response: We provided some data in the original submission showing the p53 binding induced TERRA (Fig. 2D) and histone acetylation at the 18q and 13q subtelomeres in H1299 cells with or without inducible p53 (Fig. S4). We now provide new data showing the effect
of p53 shRNA depletion in human diploid fibroblasts (new Fig. S9). We show that p53
depletion leads to an increase accumulation of γH2AX globally (Western) (Fig. S9A), and at
subtelomeric regions (ChIP assay) (Fig. S9C and D). We show that p53 depletion leads to a
loss of subtelomeric enhancer-like RNA and loss of PARD6 gene, a likely target of the 18q
subtelomere p53 enhancer function (Fig. S9B). We show a loss of subtelomeric histone
H3K27acetylation in p53 shRNA depleted diploid fibroblasts (Fig. S9C and D). We believe
that these studies help to address the concern that p53 binding and protection against
γH2AX accumulation is limited to only cancer cell lines.

2- Is the transactivation domain of p53 required for the observed phenotypes? This
experiment would allow the authors to distinguish between a secondary effect induced by
p53-responsive genes and the direct action of p53 at chromatin that is suggested by the
authors. Technically this is a simple experiment that would involve complementation of p53-
/- cells with a transactivation deficient p53 allele. Results of these experiments could
strengthen the model proposed by the authors.

Response: We agree that this is an important question. Unfortunately, we have not
been able to complete these experiments in the given time frame, and focused on the
experiments in diploid fibroblasts. We include a comment in the Discussion p18 to address
this unanswered question.

“However, future studies will be required to determine if the p53 activation
domain or any of these transcriptional co-activators are required for this process.”

3- The authors propose that different p53-interacting proteins might be required for the
p53-dependent protection of telomeres (e.g. CBP/p300, ATM, p53BP1). When possible the
authors should establish whether any of these factors is required for p53-mediated
inhibition of DNA damage accumulation at telomeres. I have suggested these experiments
since the results could provide mechanistic insight in the p53 ability to protect telomeres.
The author’s response is that by ChIP they could not detect these factors at chromosome
ends. However, as stated by the authors, negative result by ChIP does not represent a valid
reason to exclude the role of any of these factors on these processes. Down-regulation of
these proteins could help the authors test their model and provide a mechanistic insight in
this process.

Response: We agree that these mechanistic questions are important. As noted
previously, we have not been successful in ChIP assays with CBP, p300, ATM or 53BP1 at
subtelomeres, and therefore could not adequately address this question. ShRNA depletion
of these factors could be informative, but we have not had the capacity to get these
experiments completed in the allowed time. We now include a comment in the revised
Discussion p18 to address this unanswered question.

“However, future studies will be required to determine if the p53 activation
domain or any of these transcriptional co-activators are required for this process.”
Thank you for submitting your re-revised manuscript for our consideration. I have now looked through it, as well as at your responses to the previous comments and decision letter, and I am pleased to inform you that we consider all key issues addressed now. We shall therefore be happy to proceed with formal acceptance of the paper, following addressing of a few remaining editorial points as follows:

* please provide a new higher-quality/higher-resolution version of Figure 1, in which panels A-C are currently too blurry for production purposes

* please amend the manuscript text with brief "Conflict of Interest" and "Author Contribution" statements

* please adjust the in-text reference citation format according to EMBO Journal style guidelines ("aaa et al", not "aaa, bbb et al")

* please deposit ChIP-seq datasets in the appropriate public repositories and provide accession numbers in the manuscript text, as specified in our author guidelines

* the supplementary files will need altering in accordance with our new format for these types of information (see http://emboj.embopress.org/authorguide#expandedview): the easiest here is to relabel supplemental files as 'Appendix', with figures therein being labelled 'Appendix Figure S#', and tables 'Appendix Table S#'. Also, references to these files have to be changed accordingly throughout the Article text.

* please suggest 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis of a 'Synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples.

* In addition, I would encourage you to also provide an image for the Synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size is fixed to 550 pixels in width and 150-400 pixels in height. In this case, I would suggest to create a somewhat simplified version of the model in Figure 8, with rearrangements so as to snugly fit into the 550x400 pixel 'landscape' format of our synopses.

I am therefore returning the manuscript to you once more for a final round of modification, allowing you to introduce these changes. Please, don't hesitate to contact our office in case there should be any confusion around the supplementary information policies. Once we will have received your final files, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!