

The adaptive molecular landscape of reprogrammed telomeric sequences

Corresponding Author: Dr Gianni Liti

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, D'Angiolo and colleagues investigate the evolution of novel telomeric sequences by introducing human telomeric repeats (T₂AG₃) and the TLC1 gene encoding the telomerase RNA template into budding yeast. By combining mutation accumulation lines (MAL) and adaptive evolution lines (AEL), the authors elegantly follow how these "humanized" telomeres evolve over successive generations. The study convincingly shows that yeast progressively accumulate T₂AG₃ repeats, illustrating a clear transition from native to human telomeric architecture.

Importantly, the authors demonstrate that the acquisition of humanized telomeres initially compromises cellular fitness and increases genome instability, leading to elevated mutation rates. Adaptive recovery is largely achieved through two routes: attenuation of DNA-damage checkpoint activity and the duplication of chromosome XVI, where increased gene dosage of TBF1 appears to be a key driver of fitness restoration.

Overall, this work provides a compelling mechanistic and evolutionary perspective on how telomeric sequence composition shapes genome stability. The methodology is rigorous and diverse, the experimental effort substantial, and the conclusions well supported by the data. The manuscript is clearly written, well referenced, and of broad interest to the telomere, genome stability, and evolutionary biology fields. I support publication after the authors address the following points, which I believe will strengthen the manuscript and broaden its impact:

- 1) The authors state in the introduction that telomeric sequence diversification occurred "multiple times during evolution." Since plant and opisthokont telomeric sequences are highly conserved, this sentence could benefit from clarification and, ideally, a reference specifying whether these changes reflect independent evolutionary events across clades, or turnover within particular lineages such as *S. cerevisiae*. Adding specificity would strengthen the argument.
- 2) It would be helpful to highlight additional key genes (e.g., ADE2) directly in the volcano plot (Figure 1) to facilitate interpretation without needing to refer to supplementary tables. Additionally, in some supplemental figures (e.g., Supplementary Fig. 1), the font appears quite small for print; if possible, increasing font size would improve readability.
- 3) The enrichment of genes associated with ceramide biosynthesis and the cell periphery in humanized MAL strains is mentioned but not discussed. While the focus on DNA-damage response attenuation is appropriate and well explored, a brief comment on how alterations in membrane-associated pathways might relate to telomere stress or adaptive responses would be valuable, even if speculative.
- 4) Please clarify (if possible) whether increased TBF1 dosage on chromosomes other than chromosome XVI (e.g., via local duplication or ectopic integration) would be sufficient for adaptation, or whether ChrXVI aneuploidy specifically provides an additional selective advantage.
- 5) Given the genome-wide instability observed, do the authors detect changes in pericentromeric/centromeric silencing or increased frequency of neocentromeres in humanized yeast after multiple generations? Even if negative, commenting on this would be informative for the broader chromosome biology community.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The manuscript D'Angiolo et al. presents a thorough and insightful investigation into how yeast cells respond—both adaptively and non-adaptively—to the replacement of their native telomeric sequences with human-like repeats. The study builds elegantly on previous observations, reinforcing and extending our understanding that telomere humanization triggers a DNA damage response (DDR), and that attenuation of this response—through specific mutations—leads to improved cellular fitness. Importantly, the authors find an increased genome-wide mutation rate in humanized cells. Furthermore, they identify aneuploidy involving amplification of the telomere-binding protein TBF1 as an early and recurrent adaptive mechanism. With a clever experimental design combining state-of-the-art proteomics, fitness assays, and long-term evolution experiments, the authors illuminate the molecular routes available to cells facing a sudden change in telomeric repeats and the telomeric instability associated with it. Overall, this study makes a substantial contribution to our understanding of how telomeric sequences and their associated protein networks can evolve under selective pressure, offering valuable insights into the evolutionary plasticity of chromosome end maintenance.

Major Comments

- I'm unclear about the interpretation of the proteomic data presented in Figure 1g. The authors suggest these changes result from derepression of genes following Rap1 replacement with Tbf1 (line 134). However, only ADE2 appears significantly upregulated, while the other 13 genes show reduced expression compared to WT after 2 SCBs. Could the authors clarify this discrepancy?
- The authors define a “telomere humanization proteomic response” (THPR) based on proteins differentially abundant in at least two humanized MALs (line 285). I assume the intent was to capture a response to extended human telomeric repeats, distinguishing it from an acute or adaptive response. However, MALs are also undergoing adaptation (as shown by the doubling time changes in Fig. 3a), raising questions about the purity of this response. In this context, using samples at 40 SCBs might better represent early telomere-specific responses than those at 100 SCBs. Additionally, many AEL lines retain substantial human telomeric repeats after evolution. Have the authors assessed whether the THPR is still present in these lines, and to what extent? Can the authors distinguish between a physiological response (most likely more prominent in the 40SCBs, and an adaptive one? More evident in the AELs, with some potential overlap with the 100SCB samples?
- The discussion presents DDR attenuation and aneuploidy (via TBF1 amplification) as alternative adaptive strategies to humanized telomeres, potentially operating through the same mechanism (i.e., attenuating DDR and promoting faster division). The authors propose a two-step model—aneuploidy arising first, then being replaced by less costly DDR mutations—consistent with existing literature.

However, evidence for this is dispersed across the manuscript, making it difficult to track which evolved lines follow which adaptation path. A visual summary showing which lines exhibit TBF1 amplification and which have DDR mutations would clarify the narrative. If no longitudinal data per line is available, the authors should explicitly state that the model of substitution is speculative.

Minor Comments

- The four differentially expressed proteins in the humanized ancestor at 0 SCBs are referred to in Table S11, but should be Table S12.
- In line 166, the authors approximate the length of the AEL to ~400 generations. However, in the methods, they report 31 1:100 dilutions. This should correspond to 6.6 generations per transfer, resulting in ~200 generations. Is this a typo, or does the difference derive from a different estimate of the generations, and if so, which one?
- In the text and figures, serial transfers are referred to as ‘st’ (line 166); however, to avoid confusion, I believe they should be referred to with capital letters, as for the SCBs (ST for singular, STs for plural, as present in the methods).

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

This study integrates mutation accumulation (MAL) with adaptive evolution (AEL) to chart the adaptive molecular landscape triggered by telomeric sequence reprogramming. The authors document early genome instability, proteome-wide reprogramming, and fitness decline, followed by recovery via two recurrent routes: (i) increased TBF1 dosage—often through chrXVI aneuploidy and finer structural variants—and (ii) attenuating mutations in the DNA damage response (DDR). The work addresses a fundamental question—how telomere sequence changes drive long-term organismal adaptation—with a coherent multi-omics framework and notable novelty. The findings are of high conceptual interest and merit publication after additional validation.

Major concerns :

1. The data presented in Figure 2E, which shows the proportion of T2AG3 sequences across chromosomes, is particularly interesting. We noted some variability in the humanization efficiency among different clones and chromosomes, an

observation that could be further highlighted and discussed in the main text. To enhance the impact of this work, it would be helpful if the authors could elaborate on the potential biological or technical reasons behind the observed heterogeneity. Additionally, some discussion on the challenges of achieving complete humanization across an entire chromosome would be very insightful for the reader community.

2. There are certain differences between human and yeast telomere-associated proteins. Would overexpressing some human telomere proteins in yeast make the study of humanized telomeres in yeast more meaningful?

3. The authors employed two laboratory evolution protocols—mutation accumulation lines (MAL) and adaptive evolution lines (AEL)—to effectively investigate the evolution of telomere sequences. It is suggested that the authors clarify in the Discussion section the distinct mutational outcomes associated with each evolution method and provide a rationale for these differences. For instance, while the authors observed chromosome XVI aneuploidy specifically in the MAL group, other studies have indicated that aneuploidy adaptations are more commonly associated with adaptive evolution lines.

Minor concerns:

4. The key conclusions rely on assembly and quantification of terminal repeats using long reads. It is recommended that the Methods section be written in more detail.

5. The key conclusions rely on changes in doubling time; the Results section should add the calculation methods, and explain why correlations were computed on the log scale or not log scale.

6. The combination of mutation accumulation (MAL) and adaptive evolution (AEL) protocols is a powerful design that effectively distinguishes stochastic mutations from adaptive ones. However, the rationale for selecting the specific endpoints of 100 single-cell bottlenecks for MAL and 31 serial transfers for AEL is not explicitly justified. It would be important to clarify whether these thresholds were defined based on preliminary biological observations or statistical considerations.

7. Line 257 mentions that although yeast mitosis has been restored during evolution, chronological lifespan has not been restored. Mechanism behind this could be further discussed.

8. The discussion could be strengthened by connecting telomere sequence evolution with the broader concept of cross-species telomere complex compatibility. Since telomere maintenance relies on co-evolution between telomeric DNA and binding proteins, contrasting the yeast humanization model with examples from other taxa would help contextualize the evolutionary adaptability and constraints observed here.

9. Petite mutants in wild-type MALs are discarded without quantifying their incidence in humanized lines or exploring telomere–mitochondria interactions.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed my comments and revised the manuscript accordingly. The final version of the paper has improved substantially following the suggestions of all reviewers. Overall, I am satisfied with the authors' responses and recommend the manuscript for publication.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have addressed all of my comments and have made substantial efforts to improve the clarity of the numerous experimental methods employed. This work represents a significant contribution to our understanding of telomere plasticity and evolution, and the manuscript is likely to become a reference in the field.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily addressed my concerns. I recommend acceptance.

(Remarks on code availability)

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Reviewer #1 (Remarks to the Author):

In this manuscript, D'Angiolo and colleagues investigate the evolution of novel telomeric sequences by introducing human telomeric repeats (T_2AG_3) and the TLC1 gene encoding the telomerase RNA template into budding yeast. By combining mutation accumulation lines (MAL) and adaptive evolution lines (AEL), the authors elegantly follow how these “humanized” telomeres evolve over successive generations. The study convincingly shows that yeast progressively accumulate T_2AG_3 repeats, illustrating a clear transition from native to human telomeric architecture.

Importantly, the authors demonstrate that the acquisition of humanized telomeres initially compromises cellular fitness and increases genome instability, leading to elevated mutation rates. Adaptive recovery is largely achieved through two routes: attenuation of DNA-damage checkpoint activity and the duplication of chromosome XVI, where increased gene dosage of TBF1 appears to be a key driver of fitness restoration.

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1) The authors state in the introduction that telomeric sequence diversification occurred “multiple times during evolution.” Since plant and opisthokont telomeric sequences are highly conserved, this sentence could benefit from clarification and, ideally, a reference specifying whether these changes reflect independent evolutionary events across clades, or turnover within particular lineages such as *S. cerevisiae*. Adding specificity would strengthen the argument.

[R] We apologize if this was unclear. Extensive investigations to detect telomeric sequences of various taxa in published genomic sequences have led to the hypothesis that the vertebrate-like T_2AG_3 repeat is the ancestral unit from which other variants evolved, given its wide-spread conservation across multiple phylogenetic lineages, including basal branches (Fulnečková et al., 2013). Starting from this assumption, other studies have hypothesised models in which variations in the telomeric sequences originate from the gradual accumulation of mutations in the gene coding for the telomerase RNA template. Subsequently, the new telomeric repeats are accommodated by modifications of telomere-binding-proteins (Červenák et al., 2021).

[A] We rephrased this sentence in the introduction and clarified the current model of telomere sequence evolution by expanding this section (page 3, lines 50-57).

2) It would be helpful to highlight additional key genes (e.g., ADE2) directly in the volcano plot (Figure 1) to facilitate interpretation without needing to refer to supplementary tables. Additionally, in some supplemental figures (e.g., Supplementary Fig. 1), the font appears quite small for print; if possible, increasing font size would improve readability.

[R&A] We indicated the other 13 genes that are differentially expressed in the humanized ancestor at 2 SCBs in Figure 1g. Furthermore, we increased the font size in the figures to meet the figure preparation guidelines.

3) The enrichment of genes associated with ceramide biosynthesis and the cell periphery in humanized MAL strains is mentioned but not discussed. While the focus on DNA-damage response attenuation is appropriate and well explored, a brief comment on how alterations in membrane-associated pathways might relate to telomere stress or adaptive responses would be valuable, even if speculative.

[R] Mutations in ceramide biosynthesis genes (*LAC1*, *LAG1*, *ISC1*) occurred and were maintained in three independent humanized MAL. *LAC1* and *LAG1* are components of ceramide synthase, an enzyme that synthesizes ceramide from C26(acyl)-coenzyme A and dihydrosphingosine or phytosphingosine, while *ISC1* codes for inositol phosphosphingolipid phospholipase C, an enzyme that hydrolyzes complex sphingolipids to produce ceramide. All three mutations have low SIFT scores: mutations in *LAC1* (SIFT=8e⁻⁵) and *ISC1* (SIFT=0) are located in conserved regions of the protein, and the mutation in *LAG1* is a stop-gain, indicating that they are likely to induce loss-of-function in the resulting proteins (Tables S10-11). Ceramide acts as a signalling molecule and mediates cell cycle arrest, cell senescence and apoptosis, both in yeast and in mammals (Hannun, 1996; Hannun & Luberto, 2000). Moreover, ceramide can inhibit telomerase and induce telomere shortening in human cells (Ogretmen et al., 2001; Sundararaj et al., 2004). Therefore, mutations that inhibit ceramide biosynthesis are likely to have a beneficial effect on humanized yeasts and should be under positive selection. This is also in agreement with our proteomics results in which we identified downregulation of ceramide biosynthesis pathways in humanized yeasts at 40 SCBs (see response to point 7 raised by reviewer 2).

[A] We now discuss this aspect in the results section (page 17, lines 372-382).

4) Please clarify (if possible) whether increased *TBF1* dosage on chromosomes other than chromosome XVI (e.g., via local duplication or ectopic integration) would be sufficient for adaptation, or whether ChrXVI aneuploidy specifically provides an additional selective advantage.

[R] We apologize if this was unclear. We ectopically integrated an additional copy of *TBF1* in the *HO* locus on chromosome IV in a humanized strain carrying only one chromosome XVI (and one copy of *TBF1*), as illustrated in Figure 5c and explained in the methods (page 36, lines 906-930). This was sufficient to decrease the doubling time of the strain carrying this construct by about 30 minutes. Since this fitness gain was independent from a gain of copy of chromosome XVI, an increased *TBF1* dosage alone can rescue the growth in humanized yeasts. However, we cannot exclude that other genetic or epigenetic factors on chromosome XVI might also contribute to the fitness gain.

[A] We clarified in the main text (pages 20-21, lines 477-485) that our *TBF1* copy number increase results from an ectopic integration of the gene in the *HO* locus on chromosome IV, without chromosome XVI duplication.

5) Given the genome-wide instability observed, do the authors detect changes in pericentromeric/centromeric silencing or increased frequency of neocentromeres in humanized yeast after multiple generations? Even if negative, commenting on this would be informative for the broader chromosome biology community.

[R] We did not detect changes in expression of genes located near centromeres and the genes that are differentially expressed in humanized yeasts do not seem to cluster in any genomic region. Moreover, *S. cerevisiae* has point centromeres and we do not expect to observe centromere instability. We re-examined the genome assemblies of humanized yeasts derived from long-read sequencing and did not detect any cases of neo-centromere formation. On the other hand, all forms of extra chromosome XVI included a centromere that is required to ensure segregation. The assembly of Hum8 merits a separate discussion as it revealed a chromosomal rearrangement involving chromosomes V and XV. This rearrangement resulted in the internalization of chrV-R telomeres as ITS and it is stabilized by chrXV-L telomeres and its centromere (CEN15). It is unclear whether the remaining parts of chromosomes V and XV are attached to each other or constitute two independent chromosomes. The first scenario is more likely as the resulting

recombinant chromosome would be stabilized by chromosome V centromere (CEN5). The second scenario would involve the formation of a neo-centromere and is more unlikely, in fact we did not detect any extra centromeres.

[A] We added these results on page 14, lines 327-329 and in supplementary discussion 1 (page 2, lines 33-34).

Reviewer #2 (Remarks to the Author):

The manuscript D'Angiolo et al. presents a thorough and insightful investigation into how yeast cells respond—both adaptively and non-adaptively—to the replacement of their native telomeric sequences with human-like repeats. The study builds elegantly on previous observations, reinforcing and extending our understanding that telomere humanization triggers a DNA damage response (DDR), and that attenuation of this response—through specific mutations—leads to improved cellular fitness. Importantly, the authors find an increased genome-wide mutation rate in humanized cells. Furthermore, they identify aneuploidy involving amplification of the telomere-binding protein TBF1 as an early and recurrent adaptive mechanism. With a clever experimental design combining state-of-the-art proteomics, fitness assays, and long-term evolution experiments, the authors illuminate the molecular routes available to cells facing a sudden change in telomeric repeats and the telomeric instability associated with it. Overall, this study makes a substantial contribution to our understanding of how telomeric sequences and their associated protein networks can evolve under selective pressure, offering valuable insights into the evolutionary plasticity of chromosome end maintenance.

Major Comments

6) I'm unclear about the interpretation of the proteomic data presented in Figure 1g. The authors suggest these changes result from derepression of genes following Rap1 replacement with Tbf1 (line 134). However, only ADE2 appears significantly upregulated, while the other 13 genes show reduced expression compared to WT after 2 SCBs. Could the authors clarify this discrepancy?

[R&A] We agree with the reviewer that it is unlikely that Rap1p's extrusion from humanized telomeres is the reason of the downregulation of 13 genes in the humanized ancestor at 2 SCBs, as Rap1p acts mainly as a transcriptional activator. We removed that sentence.

7) The authors define a “telomere humanization proteomic response” (THPR) based on proteins differentially abundant in at least two humanized MALs (line 285). I assume the intent was to capture a response to extended human telomeric repeats, distinguishing it from an acute or adaptive response. However, MALs are also undergoing adaptation (as shown by the doubling time changes in Fig. 3a), raising questions about the purity of this response. In this context, using samples at 40 SCBs might better represent early telomere-specific responses than those at 100 SCBs. Additionally, many AEL lines retain substantial human telomeric repeats after evolution. Have the authors assessed whether the THPR is still present in these lines, and to what extent? Can the authors distinguish between a physiological response (most likely more prominent in the 40 SCBs, and an adaptive one? More evident in the AELs, with some potential overlap with the 100 SCB samples?

[R] We thank the reviewer for this suggestion. We further defined an early THPR (proteins that are differentially abundant in at least 2 humanized MAL at 40 SCBs), late THPR (proteins that are differentially abundant in at least 2 humanized MAL at 100 SCBs) and AEL response (proteins that are differentially abundant in at least 2 humanized AEL from 100 SCBs). By intersecting these responses and conducting a GO-term enrichment analysis, we found that the upregulation of ribonucleotide reductase is present in both early and late THPR and persists even after AEL. Downregulation of trehalose and glycogen metabolism persists across early and late THPR but is lost upon AEL. Interestingly, the early THPR is characterized specifically by downregulation of

phospholipid metabolism and ceramide biosynthesis, but this enrichment is lost in the late THPR. Loss-of-function mutations in ceramide biosynthesis genes (*LAC1*, *LAG1*, *ISC1*) occurred and were maintained in three independent humanized MAL (Tables S10-11, see response to point 3 raised by reviewer 1). Ceramide acts as a signalling molecule and mediates cell cycle arrest, cell senescence and apoptosis, both in yeast and in mammals (Hannun, 1996; Hannun & Luberto, 2000). Moreover, ceramide can inhibit telomerase and induce telomere shortening in human cells (Ogretmen et al., 2001; Sundararaj et al., 2004). Taken together, these results suggest that the early telomere-specific response already has an adaptive component, and downregulation or loss-of-function mutations in ceramide biosynthesis genes can rescue the growth defects induced by telomere humanization.

[A] We added a summary of these results in the main text (page 13, lines 318-326) and we extensively present them in supplementary discussion 2 (pages 2-3, lines 47-67). The early and late THPR, AEL response and their intersections, as well as their GO-term enrichments, are shown in Table S9 and Supplementary Figure 5a.

8) The discussion presents DDR attenuation and aneuploidy (via TBF1 amplification) as alternative adaptive strategies to humanized telomeres, potentially operating through the same mechanism (i.e., attenuating DDR and promoting faster division). The authors propose a two-step model—aneuploidy arising first, then being replaced by less costly DDR mutations—consistent with existing literature. However, evidence for this is dispersed across the manuscript, making it difficult to track which evolved lines follow which adaptation path. A visual summary showing which lines exhibit TBF1 amplification and which have DDR mutations would clarify the narrative. If no longitudinal data per line is available, the authors should explicitly state that the model of substitution is speculative.

[R&A] We thank the reviewer for this suggestion. We have now summarised the mutational outcomes of our 16 humanized lines during MAL and AEL by adding Figure 6. An extended version of the experimental evolution outcome can also be found in Supplementary Figure 1.

Minor Comments

9) The four differentially expressed proteins in the humanized ancestor at 0 SCBs are referred to in Table S11, but should be Table S12.

[R&A] We apologize for this typo. We revised the numeration of the supplementary tables to reflect the order in which they are cited in the manuscript. Tables S11 and S12 are now Tables S8 and S9. We now cite the correct table (S9) on page 5, line 132.

10) In line 166, the authors approximate the length of the AEL to ~400 generations. However, in the methods, they report 31 1:100 dilutions. This should correspond to 6.6 generations per transfer, resulting in ~200 generations. Is this a typo, or does the difference derive from a different estimate of the generations, and if so, which one?

[R] We tried to calculate the total number of generations based on growth rates before and after AEL but we agree with the reviewer that it is more correct to report the theoretical number of generations based on dilution factors and number of transfers, although this implies no cell death and equal fitness across the AEL.

[A] We revised our calculations and used the formula $n = \log_2(DF)$ to estimate the total number of generations for AEL, where DF =dilution factor (1:100). This gives us an estimate of 6.64 generations per serial transfer, and 206 total generations for the AEL, in agreement with the reviewer's calculations. We replaced the old value on page 8, line 172 and added the explanation for this calculation in the methods (page 33, lines 802-805).

11) In the text and figures, serial transfers are referred to as 'st' (line 166); however, to avoid confusion, I believe they should be referred to with capital letters, as for the SCBs (ST for singular, STs for plural, as present in the methods).

[R&A] We changed "scb" to "SCBs" and "st" to "STs" across the text, figures and tables.

Reviewer #3 (Remarks to the Author):

This study integrates mutation accumulation (MAL) with adaptive evolution (AEL) to chart the adaptive molecular landscape triggered by telomeric sequence reprogramming. The authors document early genome instability, proteome-wide reprogramming, and fitness decline, followed by recovery via two recurrent routes: (i) increased TBF1 dosage—often through chrXVI aneuploidy and finer structural variants—and (ii) attenuating mutations in the DNA damage response (DDR). The work addresses a fundamental question—how telomere sequence changes drive long-term organismal adaptation—with a coherent multi-omics framework and notable novelty. The findings are of high conceptual interest and merit publication after additional validation.

Major concerns:

12) The data presented in Figure 2E, which shows the proportion of T₂AG₃ sequences across chromosomes, is particularly interesting. We noted some variability in the humanization efficiency among different clones and chromosomes, an observation that could be further highlighted and discussed in the main text. To enhance the impact of this work, it would be helpful if the authors could elaborate on the potential biological or technical reasons behind the observed heterogeneity. Additionally, some discussion on the challenges of achieving complete humanization across an entire chromosome would be very insightful for the reader community.

[R] We thank the reviewer for this suggestion. Telomerase does not act at all telomeres at every cell cycle, but the probability of a telomere to be elongated increases as its length decreases. Moreover, the number of nucleotides added by telomerase is not always the same. When a telomere approaches critical length, telomerase will elongate it to restore a normal length (Teixeira et al., 2004; Wellinger & Zakian, 2012). In the case of humanized yeasts, this implies that a minimum tract of TG₁₋₃ repeats will never get eroded and replaced by T₂AG₃ repeats.

Humanized yeasts' telomeres are subject to a higher turnover, as shown in previous studies (Bah et al., 2011). This, together with the stochasticity of telomerase's action in terms of number of nucleotides added and probability of action at each single chromosome-end during the cell cycle, explains a great part of the heterogeneity that we observed.

The high turnover of humanized yeasts' telomeres also implies that they can easily reach critical length and activate DDR. This is compensated by the abundant amplifications of subtelomeric ITS and Y' elements that are reminiscent of type I survivors, which usually have short telomeres. Yeasts with telomere defects can also survive through minor amplifications of subtelomeric elements coupled with extremely long telomeric repeats, that are maintained through rolling circle replication (type II survivors) (Wellinger & Zakian, 2012). The fully humanized telomere in Hum15 TELIVR (>3 kb) is consistent with a type II survivor.

Overall, the maintenance of an internal TG₁₋₃ tract, as well as the global heterogeneity in telomere length, can be explained by the combined action of telomerase dynamics, its regulation by telomere length and alternative-lengthening-of-telomeres mechanisms.

[A] We further discussed the heterogeneity and dynamics of humanized yeasts' telomeres in the results section (pages 8-9, lines 191-198 and 212-217).

13) There are certain differences between human and yeast telomere-associated proteins. Would overexpressing some human telomere proteins in yeast make the study of humanized telomeres in yeast more meaningful?

[R&A] Previous studies have shown that the human telomerase RNA and the telomerase catalytic subunit (TERT) can be successfully expressed in yeast, they localize to the nucleus and show processivity in yeast cell extracts. However, no telomerase activity could be detected at yeast telomeres *in vivo*, meaning that it might be necessary to express additional human-specific components in order to reconstitute a functional human telomerase holoenzyme in yeast (Bah et al., 2004). To our knowledge, no studies have tried to express human telomere binding proteins (TRF1, TRF2) together with telomerase components in wild type or humanized yeasts. Given the high specificity of TRF1/TRF2, it is unlikely that their expression in wild type yeasts would lead to a functional complementation of their yeast counterparts. In humanized yeasts, the telomeric DNA is bound by Tbf1p. The simultaneous presence of Tbf1p with TRF1 and TRF2 would lead to competition for the same binding sites. The resulting telosome structure will be ultimately dictated by which proteins have the strongest affinity for the T₂AG₃ repeats.

Telomere biology is interconnected with DNA repair pathways, cell cycle regulation, mitochondrial biology and other core cellular functions. A change in telomere binding proteins would disrupt these interconnected networks leading to unpredictable fitness outcomes. Future studies expressing additional components of the telomerase holoenzyme and the shelterin complex will help to answer these questions and test the plasticity of telomere functions.

14) The authors employed two laboratory evolution protocols—mutation accumulation lines (MAL) and adaptive evolution lines (AEL)—to effectively investigate the evolution of telomere sequences. It is suggested that the authors clarify in the Discussion section the distinct mutational outcomes associated with each evolution method and provide a rationale for these differences. For instance, while the authors observed chromosome XVI aneuploidy specifically in the MAL group, other studies have indicated that aneuploidy adaptations are more commonly associated with adaptive evolution lines.

[R] The aneuploidies in MAL are less expected given the close to neutral scenario. However, under strong selection and given their relatively high occurrence rate, it is reasonable to observe them in MAL despite their relatively small population size. This has been observed also in MAL performed under rapamycin selection with repeated chromosome XII amplifications to counteract the loss of rDNA (Li et al., 2023).

[A] We clarified this interpretation in the discussion section (page 27, lines 648-653).

Minor concerns:

15) The key conclusions rely on assembly and quantification of terminal repeats using long reads. It is recommended that the Methods section be written in more detail.

[R] We annotated TG₁₋₃ and T₂AG₃ repeats in the genome assemblies by using a custom Perl script adapted from our recently published pipeline Y^{ea}ISTY (D'Angiolo et al., 2023). This script inspects the scaffolds of genome assemblies and detects single units of telomeric motifs (CA, CCA, CCCA, TG, TGG, TGGG). If units are consecutive to one another they are considered as part of the same telomeric repeats stretch and its start/end points are assigned, respectively, as the position of the first base of the first unit and the position of the last base of the last unit. Once we obtained a list of telomeric repeats stretches and their start/end points in the scaffolds, we merged the repeats which were interspaced by only 1 bp into a unique stretch by using the software mergeBed included in the suite bedtools. Then, we filtered the list to keep only the stretches longer than 10 bp and we manually inspected them to eliminate breaks that were likely due to sequencing errors. The length

of the telomeric repeats stretch was then calculated as $end-start + 1$. The interstitial stretches were annotated as ITS if they closely preceded a Y' element, while the stretches at the termini were annotated as telomeres.

[A] We split the methods sections regarding annotations of subtelomeric elements (X and Y') and annotations of telomeres and ITS, which were originally merged in a single paragraph. We added a detailed description of the approach we used to annotate telomeres and ITS from long-read genome assemblies (page 42, lines 1096-1110).

16) The key conclusions rely on changes in doubling time; the Results section should add the calculation methods, and explain why correlations were computed on the log scale or not log scale.

[R] In order to get a comprehensive view of how telomere humanization impacts yeast growth, we used two complementary approaches. First, we measured growth in the same medium used for our MAL and AEL (Leucin/Tryptophan dropout) by monitoring the optical density in liquid medium over a 72 hours timespan in a TECAN plate reader. We derived the doubling time from the resulting data by using the software PRECOG and averaging the values of three biological replicates per sample. In PRECOG, the doubling time is calculated as the mean of the five highest slopes in the exponential growth phase (Fernandez-Ricaud et al., 2016).

Second, we asked whether the impact of telomere humanization would be different in other conditions that were not used in our experimental evolution. Given the high number of conditions tested we used the high-throughput and automated microbial phenomics platform Scan-o-matic, which monitors colony sizes on agar plates using desktop scanners and derives growth curves from these images. In Scan-o-matic, the doubling time is calculated as the maximum slope of the growth curve, using five timepoints for a local regression (Zackrisson et al., 2016).

The impact of telomere humanization on growth combined with the impact of multiple conditions can result in very different doubling time values across the conditions and samples, making it hard to appreciate the effect of each environment in a single plot. To improve visualization and clarity, we expressed the absolute values as relative doubling time respect to a common reference strain (wild type ancestor at 2 SCBs) and we converted them to a logarithmic scale.

[A] We clarified in the results section that we used two different approaches to measure growth and we briefly explained them (page 12, lines 259-269). We included more details on how we calculated the doubling time in the two approaches and we justified our choice of showing part of the growth data in logarithmic scale in the caption of Figure 3. We improved the clarity of the relative methods sections and we changed the title from "Estimation of the growth performance in liquid medium" to "Estimation of the growth performance in Leucine/Tryptophan dropout" to better clarify that this paragraph refers to the first approach.

17) The combination of mutation accumulation (MAL) and adaptive evolution (AEL) protocols is a powerful design that effectively distinguishes stochastic mutations from adaptive ones. However, the rationale for selecting the specific endpoints of 100 single-cell bottlenecks for MAL and 31 serial transfers for AEL is not explicitly justified. It would be important to clarify whether these thresholds were defined based on preliminary biological observations or statistical considerations.

[R] We made different considerations to plan the duration of our MAL and AEL, and these are explained below:

MAL - One of the goals of our study was to characterize the long-term impact of telomere humanization on organismal fitness, including growth, lifespan and mutation rate. To plan the duration of our MAL, we considered *S. cerevisiae*'s mutation rate and the number of generations needed to substitute yeast telomeric repeats with humanized ones. Previous studies showed that the

replacement of TG₁₋₃ with T₂AG₃ repeats is gradual and only reaches a steady state after 1200 generations (Bah et al., 2004), hence we wanted our MAL to go beyond that number of generations. Classical approaches to measure mutation rates in microbes use mutation accumulation lines (MAL) in which cells are propagated through SCBs to minimize selection. Since *S. cerevisiae* has very low base substitution rates (10^{-9} - 10^{-10} /bp/generation) (Barrick & Lenski, 2013; Sharp et al., 2018; Tattini et al., 2019) it is essential to propagate cells for a long time to obtain enough mutations for statistical comparisons. Thus, we initially planned our MAL to last for at least 2000 generations. Assuming that yeasts perform ~20 generations at every bottleneck, our minimal MAL duration was 100 SCBs.

AEL - The duration of AEL experiments is usually between few hundreds to a thousand generations (LaBar et al., 2020). Previous AEL carried out in our laboratory consisted of ~15 serial transfers, thus that number was taken as our minimum baseline (Li et al., 2023; Vázquez-García et al., 2017). Since the fitness defect caused by humanized telomeres was very pronounced, we doubled the number of serial transfers to 30 to have a higher chance of recovering adapted lines. At 31 STs, we measured the growth rate of our evolved lines and their ancestors and we found that our humanized lines had improved their growth, with some of them reaching the same growth rate as the wild types. Thus, we stopped AEL at that point.

[A] We clarified in the methods how the thresholds of 100 SCBs and 31 STs were chosen (pages 32-33, lines 768-772 and 798-802).

18) Line 257 mentions that although yeast mitosis has been restored during evolution, chronological lifespan has not been restored. Mechanism behind this could be further discussed.

[R] In both AEL and MAL, we let the cells grow for either 2 or 3 days, transferred them to another agar plate or flask and let them grow again. During these protocols, the maximum timespan that yeasts spent between cycles was 3 days. After 3 days, both humanized and wild type yeasts had reached stationary phase but their viability had not started to decrease yet. Thus, our transfer regime imposes that growth rate is constantly under selection during AEL, while there is no selection on chronological lifespan.

Previous literature has shown that humanized telomeres tend to be shorter and are subject to higher turnover than native ones. Additionally, humanized yeasts cannot grow without telomerase (Bah et al., 2011). In this context, an increase in growth rate caused by the inactivation of cell cycle checkpoints (either through point mutations in DDR genes or *TBF1* amplification and inhibition of DDR) might further exacerbate the replication stress and impose a constraint on lifespan due to limiting telomere length, higher genome instability or other yet unknown factors elicited by telomere dysfunction. This is in line with the observation that humanized yeasts promptly improve their growth but also shorten their lifespan upon the occurrence of chrXVI aneuploidy or point mutations in DDR components (Figures 4d and 5b, Supplementary Figures 4a and 7e).

[A] We now state in the results section (page 12, line 280) that our evolutionary regimes do not impose any selection on chronological lifespan, explaining why this shortens upon MAL and AEL. Moreover, we discussed potential explanations for the tradeoff between growth and lifespan that we observed upon DDR mutations and *TBF1* amplification (page 27, lines 628-634).

19) The discussion could be strengthened by connecting telomere sequence evolution with the broader concept of cross-species telomere complex compatibility. Since telomere maintenance relies on co-evolution between telomeric DNA and binding proteins, contrasting the yeast humanization model with examples from other taxa would help contextualize the evolutionary adaptability and constraints observed here.

[R] We thank the reviewer for this suggestion. The concept of cross-species telomere complex compatibility can be viewed in terms of the possibility to switch telomeric sequences between species or express a functional telomerase across species.

Yeasts can tolerate modifications in their telomeric repeats: *S. cerevisiae* strains with mutations in their telomerase RNA template were generated by (Lin et al., 2004) although they showed growth defects and telomere length dysregulation. *S. cerevisiae* yeasts with humanized telomeres are viable although they have fitness defects (Henning et al., 1998). More recently, (Červenák et al., 2025) replaced the telomeric repeats of *Yarrowia lipolytica* with those from human, *Yarrowia yakushimensis* or *Candida hispaniensis* and showed that the new telomeric repeats were readily accumulated at its telomeres. A variant telomeric repeat has been identified also in a human family (Hinchie et al., 2024).

The telomerase catalytic subunit (TERT) and its associated RNA are sufficient for telomere synthesis *in vitro*. Previous studies have examined the compatibility between these two components across species. The probability of functional complementation is higher when taxa are phylogenetically close. For example, telomerase RNAs can be successfully swapped between the ciliates *Glaucoma chattoni* and *Tetrahymena thermophila*, and the rabbit telomerase RNA is compatible with the human TERT (Bhattacharyya & Blackburn, 1997; Xiang et al., 2000). Instead, there is only partial complementation between mouse and human: while the mouse telomerase RNA cannot function with human TERT, the human telomerase RNA can function with mouse TERT (Beattie et al., 1998; Boklan et al., 2002; Martín-Rivera et al., 1998). A partial complementation is observed and between yeast and human: in fact, the human telomerase RNA and TERT can be successfully expressed in yeast, they localize to the nucleus and show processivity in yeast cell extracts. However, no telomerase activity could be detected at yeast telomeres *in vivo* (Bah et al., 2004).

[A] We strengthened the discussion by describing the cross-species telomere complex compatibility in terms of the possibility to switch telomeric sequences between species or express a functional telomerase across species (page 28, lines 663-685).

20) Petite mutants in wild-type MALs are discarded without quantifying their incidence in humanized lines or exploring telomere–mitochondria interactions.

[R] We thank the reviewer for this suggestion and we agree that exploring telomere–mitochondria interactions is exceptionally interesting. Telomeres and mitochondrial biology are closely connected and telomere dysfunction is known to affect mitochondrial metabolism through the transcriptional dysregulation of mitochondrial biogenesis pathways (Finkel, 2011; Robin et al., 2020; Sahin et al., 2011). We carried out initial experiments on our MAL to investigate the impact of telomere humanization on mitochondrial functions. Of note, we found a higher *petite* formation rate in humanized than in wild type yeasts, that seems in contrast with what occurred in our MAL. It is possible that by stochasticity we did not pick any humanized *petite* while we picked multiple wild type *petite*. Another likely explanation is that *petite* colonies disappear in humanized yeasts, especially at later timepoints, due to the fact that humanized yeasts already have growth defects and the lack of mitochondrial DNA might simply be lethal in this background. The observation that *petite* rate decreases from 60 SCBs in humanized yeasts while it remains constant for wild types seems in line with this explanation. However, we cannot exclude that the *petite* rescue might be due to chromosome XVI aneuploidy, as it happened with the rescue of mitotic growth.

We plan to expand the scope of these experiments and gain a comprehensive view of the interplay between humanized telomeres and mitochondria in a follow-up work. Our preliminary results are still unpublished but we make them available in this response letter.

[A] We investigated whether telomere humanization had an effect on mitochondrial biology by measuring several mitochondrial parameters. First, we tested whether telomere humanization

influenced the rate of *petite* colony formation (Lipinski et al., 2010). Indeed, humanized yeasts generated a higher percentage of *petite* colonies than wild types when plated on YPG medium. This effect was already present at 20 SCBs but improved at later timepoints in MAL (**Figure a**).

Second, we estimated the mitochondrial DNA copy number by analysing coverage along three mitochondrially-encoded genes: *COX2*, *COX3* and *ATP6*, as previously described (De Chiara et al., 2020). While starting from similar copy numbers at 2 SCBs (~ 2), humanized yeasts increased their mtDNA copies during MAL to a final average of ~ 3 copies, whereas wild types did not change (two-tailed Wilcoxon test, $p=0.0002$ at 100 SCBs) (**Figure b**).

Third, mitochondrial dysfunction is usually characterized by an increase of reactive oxygen species (ROS) due to malfunctioning of the electron transport chain. We measured the amount of superoxide anion ($O_2^{\bullet-}$) in humanized and wild type lines, by staining with dihydroethidium followed by flow cytometry. We found a clear difference in the amount of superoxide between the two genetic backgrounds, with humanized yeasts having significantly higher superoxide than wild types (two-tailed Wilcoxon test, $p=0.04$) (**Figure c**).

Finally, we measured mitochondrial activity and mass by double staining with Mitotracker Red and Mitotracker Green, respectively, followed by flow cytometry. Humanized yeasts had lower mitochondrial activity (two-tailed Wilcoxon test, $p=0.007$), but higher mitochondrial mass (two-tailed Wilcoxon test, $p=1.5e^{-5}$) than wild types (**Figures d-e**). These apparently contrasting results may be explained by the fact that replication of dysfunctional mitochondria results in higher mitochondrial mass but does not improve their activity. These results are also in agreement with our estimation of mtDNA copy number from whole-genome sequencing and with an increase in ROS due to respiration defects. Overall, these experiments show that telomere humanization causes mitochondrial dysfunction, and the increase in mitochondrial DNA and mass is likely a compensation mechanism to cope with a defective respiration.

[Figure Redacted]

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my comments and revised the manuscript accordingly. The final version of the paper has improved substantially following the suggestions of all reviewers. Overall, I am satisfied with the authors' responses and recommend the manuscript for publication.

[R] Thank you.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my comments and have made substantial efforts to improve the clarity of the numerous experimental methods employed. This work represents a significant contribution to our understanding of telomere plasticity and evolution, and the manuscript is likely to become a reference in the field.

[R] Thank you.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed my concerns. I recommend acceptance.

[R] Thank you.

Proposed changes to previous response letter

20) Petite mutants in wild-type MALs are discarded without quantifying their incidence in humanized lines or exploring telomere–mitochondria interactions.

[R] We thank the reviewer for this suggestion and we agree that exploring telomere–mitochondria interactions is exceptionally interesting. Telomeres and mitochondrial biology are closely connected and telomere dysfunction is known to affect mitochondrial metabolism through the transcriptional dysregulation of mitochondrial biogenesis pathways. We carried out initial experiments on our MAL to investigate the impact of telomere humanization on mitochondrial functions. Of note, we found a higher *petite* formation rate in humanized than in wild type yeasts, that seems in contrast

with what occurred in our MAL. It is possible that by stochasticity we did not pick any humanized *petite* while we picked multiple wild type *petite*. Another likely explanation is that *petite* colonies disappear in humanized yeasts, especially at later timepoints, due to the fact that humanized yeasts already have growth defects and the lack of mitochondrial DNA might simply be lethal in this background. The observation that *petite* rate decreases from 60 SCBs in humanized yeasts while it remains constant for wild types seems in line with this explanation. However, we cannot exclude that the *petite* rescue might be due to chromosome XVI aneuploidy, as it happened with the rescue of mitotic growth.