nature portfolio

Peer Review File

The de novo design and synthesis of yeast chromosome XIII facilitates investigations on aging

Corresponding Author: Professor Weiren Huang

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript from Zhou et al. demonstrates the synthesis and construction of yeast chromosome XIII according to the Sc2.0 design principles, as part of the consortium's ongoing collaborative efforts to create a synthetic yeast genome. After briefly introducing the synthetic chromosome, the manuscript focuses on a serendipitous "side effect" of PCRtagging, which altered expression of RRN9, leading to extended cellular lifespan of the yeast. The researchers use the SCRaMbLE system, in combination with high-throughput screening, to identify various genomic rearrangements affecting lifespan. The elegant use of SCRaMbLE to generate new functions for studying a fundamental biological trait as aging is refreshing and makes the manuscript suitable for Nature Communications.

In its current state, the manuscript seems rushed and will benefit from thorough reading. Main Figure 3 is mislabelled and the gene map in Sup Fig 3a seems unfinished.
In several instances, a word is missing or rephrasing is needed, e.g. line 189 should read "extended lifespan compared to its parental…".

Comments
The initial characterisation of synXIII (Fig 2) should include growth curves of relevant strains in liquid medium, and calculation of doubling time.

Altered expression levels of RRN9 (as well as 20 other targets and rearrangements) increase the cellular lifespan. Does this increase in lifespan come at a cost, e.g. increased generation time? Besides the population doubling time in liquid media (see above) the generation time must be possible to determine on single cell level from your microfluidic chip experiment. Have previous reports pointed towards a correlation between replication lifespan and generation time in yeast? Increased lifespan could, put simply, be caused by increased generation time that causes less stress for the dividing cell, somewhat analogous to starvation increasing lifespan in some higher organisms. Please discuss and relate to your finding that genes involved in translation are overrepresented in mutants with increased cellular lifespan (Fig 4c).

138, A detailed comparison between the designed and the produced SynXIII chromosome map should be supplied, highlighting the differences.
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278, blue rather than green (green is relative lifespan). Should this be up/down-regulated or over/under-represented?
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341, please specify which protein complex
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Reviewer #2

(Remarks to the Author)
This is an interesting manuscript that demonstrate how synthetic genomes can help to shed new light into important biological process, in this case replicative life span. I find very interesting the RRN9 synonymous mutation that extend life span (by changing its expression level). There is a long debate on phenotypic impact of synonymous mutation and this can be a very interesting case.

Technical comments:

- Some of the RLS data should be validate with the classical micro manipulator approach, e.g. including the synonymous mutation reconstructed in the BY and another unrelated background
- The phenotypes in figure 1b are not quantitative, why not measuring growth curves in HT way with many replicas and statistical analysis? What is the resolution to detect phenotypic changes with this approach?
- Also, figure 1c RLS should be repeated with the classic micromanipulator with an adequate number of mother tested (e.g. 80 per strain)
- The authors perform different omics, including genomics. They show changes in the chromosome XIII. Have they looked if something also has happened to the rest of the genome? What about the rDNA copy number? Would be good to see these data, and rule out off-target effects contributing to the RLS.

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Thank you for the revised manuscript. The authors have answered most of the raised questions sufficiently and conducted additional experiments where appropriate. I therefore find the manuscript mature for publication in Nature Communications once the following comments are addressed:

1) To avoid confusion, please stick to only one of the following terms: reproductive/replicative/cellular lifespan.

2) For the 21 mutants with increased reproductive lifespan, I appreciate the new data on generation time and the additional discussion. I'd appreciate to also see a plot of the chronological lifespan of the 21 mutants (relative to the original synXIII) for a comprehensive overview of relationships between the generation time, reproductive lifespan, and chronological lifespan of your mutants.

3) Line 294: Still inconsistent: If you write "noncognate UTR" in line 293, then line 294 should be "CDS-noncognate UTR".

Reviewer #2

(Remarks to the Author)
The authors have largely dismiss my comments. I think the way fitness is measured with simple spot assay inspected by eyes is not quantitative at all. This is the current state of the art when you want to measure yeast growth and fitness:

https://link.springer.com/protocol/10.1007/978-1-0716-2257-5_21

Similarly, validating some of their results with the standard RLS protocol would have make the paper stronger.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for the final revisions. My questions and concerns have been addressed and I find the manuscript mature for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
I'm happy with the revised paper and the inclusion of the additional experiments performed (colony size and daughter cells micro manipulation) that confirm the cell growth and RLS findings.

We extend our deepest gratitude to all the reviewers for their insightful and detailed suggestions. Your feedback has been instrumental in enhancing the quality of our work. In the following section, presented in blue, we offer point-by-point responses to each of the reviewers' comments.

Reviewer #1 (Remarks to the Author):

The manuscript from Zhou et al. demonstrates the synthesis and construction of yeast chromosome XIII according to the Sc2.0 design principles, as part of the consortium's ongoing collaborative efforts to create a synthetic yeast genome. After briefly introducing the synthetic chromosome, the manuscript focuses on a serendipitous "side effect" of PCRtag, which altered expression of RRN9, leading to extended cellular lifespan of the yeast. The researchers use the SCRaMbLE system, in combination with high-throughput screening, to identify various genomic rearrangements affecting lifespan. The elegant use of SCRaMbLE to generate new functions for studying a fundamental biological trait as aging is refreshing and makes the manuscript suitable for Nature Communications.

In its current state, the manuscript seems rushed and will benefit from thorough reading. Main Figure 3 is mis-labelled and the genemap in Sup Fig 3a seems unfinished.

In several instances, a word is missing or rephrasing is needed, e.g. line 189 should read "extended lifespan compared to its parental…

Response 1：We sincerely appreciate your positive evaluation of our work and helpful suggestions. Your recognition of the novel contributions our work makes to the field of synthetic biology is deeply valued, particularly in the construction of yeast chromosome XIII. We apologize for the oversights and appreciate your detailed feedback, which has guided significant revisions to enhance the clarity and completeness of the manuscript.

The manuscript has undergone an additional round of thorough proofreading. Regarding your observation of Main Figure 3 being mislabeled, we have corrected this error and ensured that all figures are accurately labeled to correspond with their descriptions in the text. Similarly, we have thoroughly revised Supplementary Figure 3a to complete the gene map, ensuring that it accurately reflects the data and supports the narrative of our findings. We have corrected the missing word in line 195, "extended lifespan compared to its parental strain. Similar corrections and rephrasing have been made throughout the manuscript to improve readability and precision.

Comments

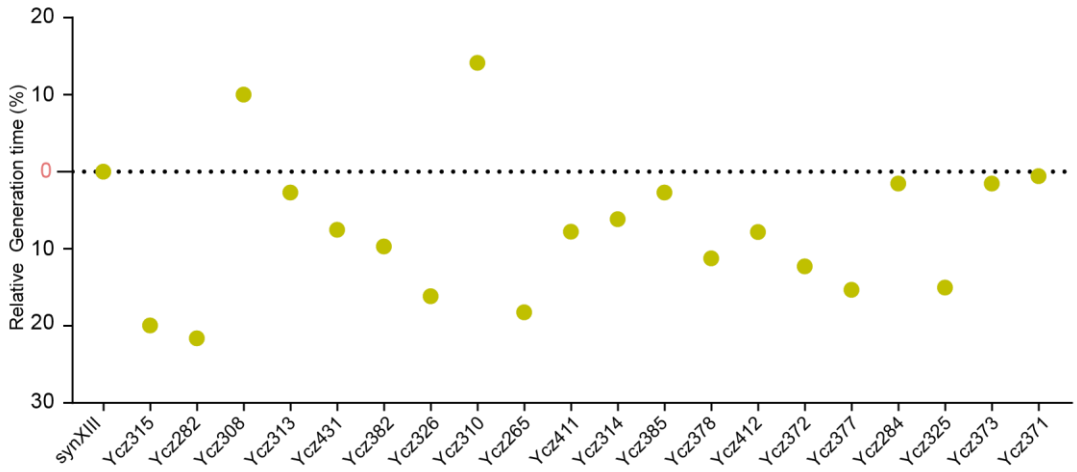
The initial characterisation of synXIII (Fig 2) should include growth curves of relevant strains in liquid medium, and calculation of doubling time.

Response 2：Thank you for your suggestion. We have updated Fig 2 (in Line 163, 176 -178) to include growth curves for the relevant strains cultured in YPD medium in Fig 2b. Additionally, we have provided the calculations for their doubling times in Fig 2c. These additions will help in better understanding the growth characteristics of the initial synXIII strain under study.

Altered expression levels of RRN9 (as well as 20 other targets and rearrangements) increase the cellular lifespan. Does this increase in lifespan come at a cost, e.g. increased generation time? Besides the population doubling time in liquid media (see above) the generation time must be possible to determine on single cell level from your microfluidic chip experiment.

Response 3：Thank you for your constructive suggestions. To follow the reviewer's suggestion, we meticulously analyzed the generation times of 20 SCRaMbLEd strains in our study, including the RRN9 strain.

Generation time refers to the duration it takes for a cell to complete one daughter cell producing. In our study, the long-lived strains (RRN9 variant strain as well as 20 other targets and rearrangements) increased the lifespan which means they produced more daughter cells compared with their corresponding parental strain. Based on our microfluidic chip experiment, we recorded all the generation times within the whole life of each cell, total 40 cells for a strain. We then statistically evaluated generation times of each strain, and found that the majority of SCRaMbLEd strains (17 out of the 20) showed shorter median generation times compared to the original synXIII strain, while the remaining three strains exhibited similar and increased generation times (See Figure below). These results indicated different underlying mechanisms are used to extend lifespan. We have incorporated relevant descriptions in Supplementary Figure 7 in line 793 - line 796. Specifically, the RRN9 strain also exhibited a slight decrease in generation time, which could indicate a unique adaptation mechanism within this genetic background. And this part will be further studied in our future work..



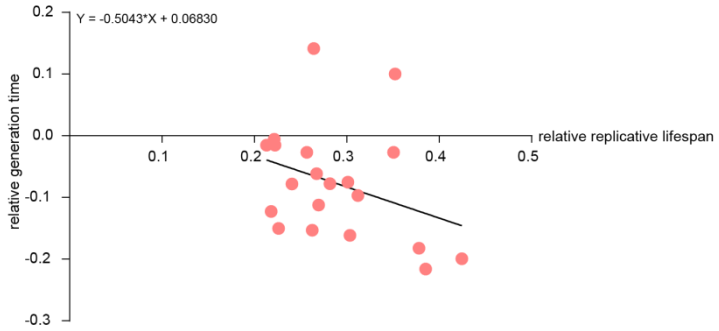
Have previous reports pointed towards a correlation between replication lifespan and generation time in yeast? Increased lifespan could, put simply, be caused by increased generation time that causes less stress for the dividing cell, somewhat analogous to starvation increasing lifespan in some higher organisms. Please discuss and relate to your finding that genes involved in translation are over-represented in mutants with increased cellular lifespan (Fig 4c).

Response 4: Thank you for your insightful suggestion. Aging in yeast can be determined by monitoring time-dependent mortality and loss of functions such as reproduction, which called chronological lifespan or replicative lifespan that defined as the number of daughter cells that are generated by individual mother cells. Starvation is recognized in eukaryotic cells to regulate lifespan from yeast to mammals. This model was usually used for chronological lifespan analysis. Longer chronological lifespan is indeed associated with longer generation time (PMID:16304601; PMID:28357278) since that chronological lifespan extension occurs when cell cycle progression is arrested or slowed down as a consequence of compromised proliferative signaling pathways or conditions of osmotic, oxidative or replicative stress. For replicative lifespan, a longer replicative lifespan is usually considered associating with a shorter generation time, such as *fob1Δ* (PMID: 22498653). A longer generation time indicates a greater division pressure, which is typically associated with a shorter lifespan. This suggests that the senescent phenotype, as manifested by lengthened generation time, is a dominant feature in yeast cells (PMID:2698814). As reported in the study (PMID: 22498653) that higher ROS levels lead to more DNA damage in the older cells which made the cell longer generation time. In the study (PMID: 25723167), we found that the inactivation

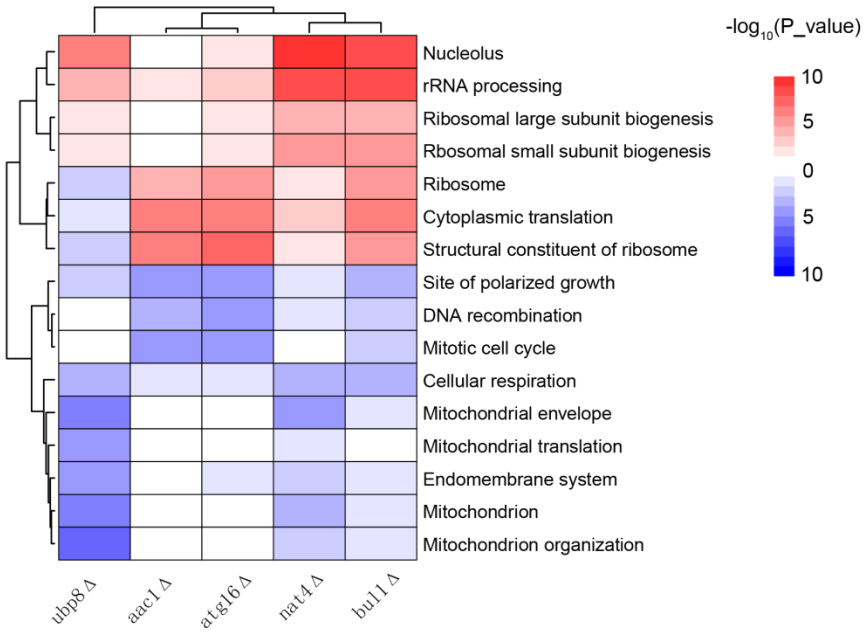
of telomerase early on caused cellular replication stress, leading to a longer generation time and a shorter replicative lifespan. After knocking out sml1, both the cell generation time and lifespan returned to normal.

In our study, we focused on studying replicative lifespan. We observed the mean generation time of all 20 long-lived SCRaMbLEd strains (See above). The results exhibited 17 out of strains show shorter mean generation time compared to synXIII. The scatter plot of generation time and replicative lifespan indicating a weak negative correlation, as shown in Figure A below. It suggested different mechanisms to extend cell lifespans. In main Fig 4c, the longer-lived strains exhibited a upregulation in genes that encode cellular components of ribosomes, rRNA processing, nucleolus and cytoplasmic translation. The enhanced ribosomal function may contribute to lifespan extension by altering protein synthesis patterns, which can increase stress resistance and improve protein homeostasis (PMID:11292874). Improved protein homeostasis enables cells to better maintain and repair themselves, thereby potentially extending lifespan (PMID:19797661). In our analysis, we identified six over-represented mutant genes—*UBP8, AAC1, NAT4, BUL1* and *ATG16*—whose null mutants exhibited lifespan extension (see Supplementary Figure 6). Each of these null mutants also demonstrated enhanced ribosomal function, as illustrated in Figure B below, aligning with the findings presented in Fig 4c. We have incorporated relevant descriptions and discussions discussion section based on our findings in line 369-line 373 and Supplementary Figure 9 in line 802-line 806.

A



B



138, A detailed comparison between the designed and the produced SynXIII chromosome map should be supplied, highlighting the differences.

Response 5: Thank you for your suggestion. In response, we have added a detailed comparison of the designed and produced synXIII chromosome maps update in Fig. 1a, highlighting the key differences, as outlined in lines 141 - line 143 of the results section.

138, Table S2 is Sup Dataset 3 or 4?

Response 6: We apologize for the confusion. Table S2 corresponds to the table labeled "Chromosome version used in this paper", which is located after the supplemental figures at the end of the main text, specifically at line 814.

148, Where is the purple?

Response 7: We apologize for the mislabeling. We have corrected “purple” to “blue” to align with the legend in Fig. 1b in Line 151.

189, “extended lifespan compared to its parental…”

Response 8: Thank you for pointing out the phrasing. We have corrected it accordingly in line 194.

203, Format gene map in Sup Fig 3a.

Response 9： We have carefully reviewed and revised the figure in Line 746 to address your concerns. The updated version now includes annotations and improved layout to enhance readability and accuracy of the data presented.

217, Fig 3

Response 10: We have made the correction accordingly in line 222

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278, blue rather than green (green is relative lifespan). Should this be up/down-regulated or over/under-represented?

Response 11：Thank you for the suggestion regarding the color coding used in our manuscript. We have updated the legend, replacing the green with blue to avoid any confusion, as blue correctly signifies down-regulation in our text. Additionally, we have clarified in the manuscript that the indicated genes are down-regulated rather than over- or under-represented to ensure that our terminology is precise and consistent with the study's findings.

289, CDS-Noncognate_UTR

Response 12: We have corrected the term "CDS-Noncognate_UTR" accordingly and highlighted the change in yellow in line 293.

303, “a large duplication”

Response 13: We have corrected accordingly in line 307.

333, recoding

Response 14: We have corrected accordingly and highlighted the change in yellow.

341, please specify which protein complex

Response 15: We have clarified this by specifying "UAF" in the text at line 346 to ensure accurate understanding and context and highlighted the change in yellow.

351, module or models?

Response 16: The correct term is "models." We have corrected accordingly and highlighted the change in yellow in line 379.

Reviewer #2 (Remarks to the Author):

This is an interesting manuscript that demonstrate how synthetic genomes can help to shed new light into important biological process, in this case replicative life span. I find very interesting the RRN9 synonymous mutation that extend life span (by changing its expression level). There is a long debate on phenotypic impact of synonymous mutation and this can be a very interesting case.

Response 1：Thank you for your positive remarks and for highlighting the significance of our findings with respect to the RRN9 synonymous mutation and its impact on replicative lifespan. We agree that this case presents an intriguing example of how synonymous mutations can have phenotypic effects, contributing to the ongoing debate in the field. Our study demonstrates that even subtle changes at the genetic level can lead to significant biological consequences by altering gene expression. We have updated our discussion on this point to further explore the implications of synonymous mutations in light of recent studies and theoretical considerations.

Technical comments:

- Some of the RLS data should be validate with the classical micro manipulator approach, e.g. including the synonymous mutation reconstructed in the BY and another unrelated background

Response 2: We thank the reviewer for their insightful comments. Our co-author, Zhengwei has contributed to invention of the first-generation gradient chips and second-generation island-type chips. These innovations marked substantial improvements over the traditional micro-manipulator approaches. Notably, similar technologies have been independently developed and validated by other research groups, including those led by Luke P. Lee and Lidong Qin, underscoring the robustness and versatility of these platforms in biological research (PMID: 22421136, PMID: 23144860, PMID: 26170317).

Recent reviews, such as those by Matt Kaeberlein and Hao Nan, have recognized these microfluidic

chips as significant breakthroughs in longevity studies (PMID: 27015709, PMID: 33880425). This technology has enabled us to conduct precise and reproducible experiments, confirming the longevity-promoting effects in human aging cell lines and mouse lifespan studies (PMID: 31773901, PMID: 32157780, PMID: 35662728).

To address your concerns and enhance clarity, we have revised the manuscript to include a detailed description of our methodologies in Lines 336-337. This amendment ensures that our experimental methods are transparent and comprehensible, reinforcing the reliability and innovativeness of our findings.

- The phenotypes in figure 1b are not quantitative, why not measuring growth curves in HT way with many replicas and statistical analysis? What is the resolution to detect phenotypic changes with this approach?

Response 3: Thank you for your insightful suggestion. We recognize the benefits of using high-throughput (HT) growth curves with multiple replicates and detailed statistical analysis for a quantitative evaluation of phenotypes. This method could indeed provide a more sensitive detection of subtle, dose-dependent phenotypic changes.

However, in the context of the Sc2.0 project, which is focused on synthesizing chromosome XIII (synXIII), our main goal is to confirm that the synthetic chromosomes maintain comparable fitness levels to the wild-type strains (BY4741 or BY4742). For this reason, we employ standardized viability testing protocols that have been widely accepted within the synthetic biology community. This standardization ensures the consistency needed for comparing fitness effects across various synthetic chromosomes developed by the Sc 2.0 consortium, thus advancing our understanding of their biological functions. It also aids in the integration of all synthetic chromosomes into a single yeast cell—a critical aim for the later phases of the Sc 2.0 project.

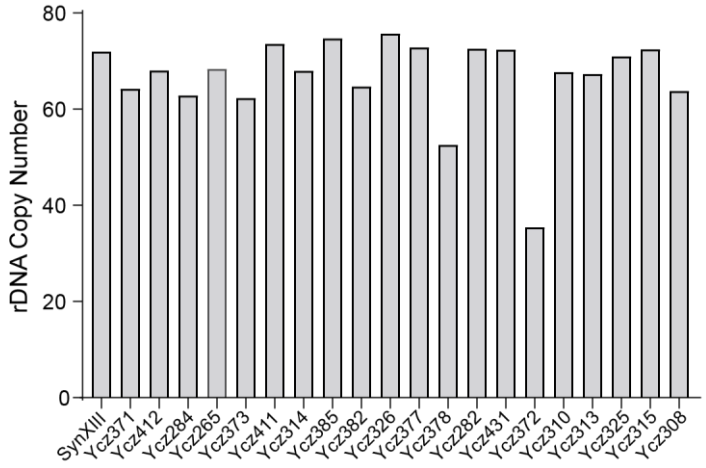
Our current testing methodology aligns with protocols used in previous studies of other synthetic chromosomes (referenced: PMID: 28280153, 24674868, 28280149). Nevertheless, we are open to considering adopting HT growth curve analysis in future studies to enhance our insights into phenotypic variations.

- Also, figure 1c RLS should be repeated with the classic micromanipulator with an adequate number of mother tested (e.g. 80 per strain)

Response 4：We appreciate the suggestion. As mentioned above, in our study detailed in Figure 1c, we conducted three independent replicates, each with 40 cells per strain, totaling 120 cells examined. This number was sufficient to achieve statistical significance and detect meaningful differences in lifespan. We are grateful upon the peer feedback.

- The authors perform different omics, including genomics. They show changes in the chromosome XIII. Have they looked if something also has happened to the rest of the genome? What about the rDNA copy number? Would be good to see these data, and rule out off-target effects contributing to the RLS.

Response 5：Thank you for your insightful suggestions. We further validated our findings by analyzing the entire genome of 20 SCRaMbLEd strains and did not find any specific functional variants such as SV, SNV, or indels. Additionally, we expanded our genomic analysis to evaluate the rDNA copy number in all SCRaMbLEd strains. The updated results, now included in the revised manuscript, show that 18 of the analyzed strains maintain rDNA copy numbers similar to the original synXIII. Unexpectedly, strains Ycz372 and Ycz378 exhibited a decrease in rDNA copy number. Previous research, such as the study by Manuel Hotz et al. (PMID: 35394872), has demonstrated a significant positive correlation between rDNA copy number and replicative lifespan. Therefore, the reduced rDNA copy number in Ycz372 and Ycz378 likely contributes to a shortened lifespan in these strains. This observation suggests that the changes observed in these two strains could be attributed to rearrangements in synthetic chromosome XIII. By expanding our genomic scrutiny to the entire genome, we have ensured that the phenotypic changes observed are directly related to the modifications within *synXIII*, thereby minimizing the impact of off-target effects on our results. We have incorporated this information into the third paragraph of discussion section (line 358-line 372) and Supplementary Figure 7 in line 791 - line 794 to clearly exhibit our findings.


rDNA Copy Number
80
60
40
20
0
SynXIII
Ycz371
Ycz412
Ycz284
Ycz265
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Ycz314
Ycz385
Ycz382
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We extend our deepest gratitude to all the reviewers for their insightful and detailed suggestions. Your feedback has been instrumental in enhancing the quality of our work. In the following section, presented in blue, we offer point-by-point responses to each of the reviewers' comments.

Reviewer #1

Thank you for the revised manuscript. The authors have answered most of the raised questions sufficiently and conducted additional experiments where appropriate. I therefore find the manuscript mature for publication in Nature Communications once the following comments are addressed:

1) To avoid confusion, please stick to only one of the following terms: reproductive/replicative/cellular lifespan.

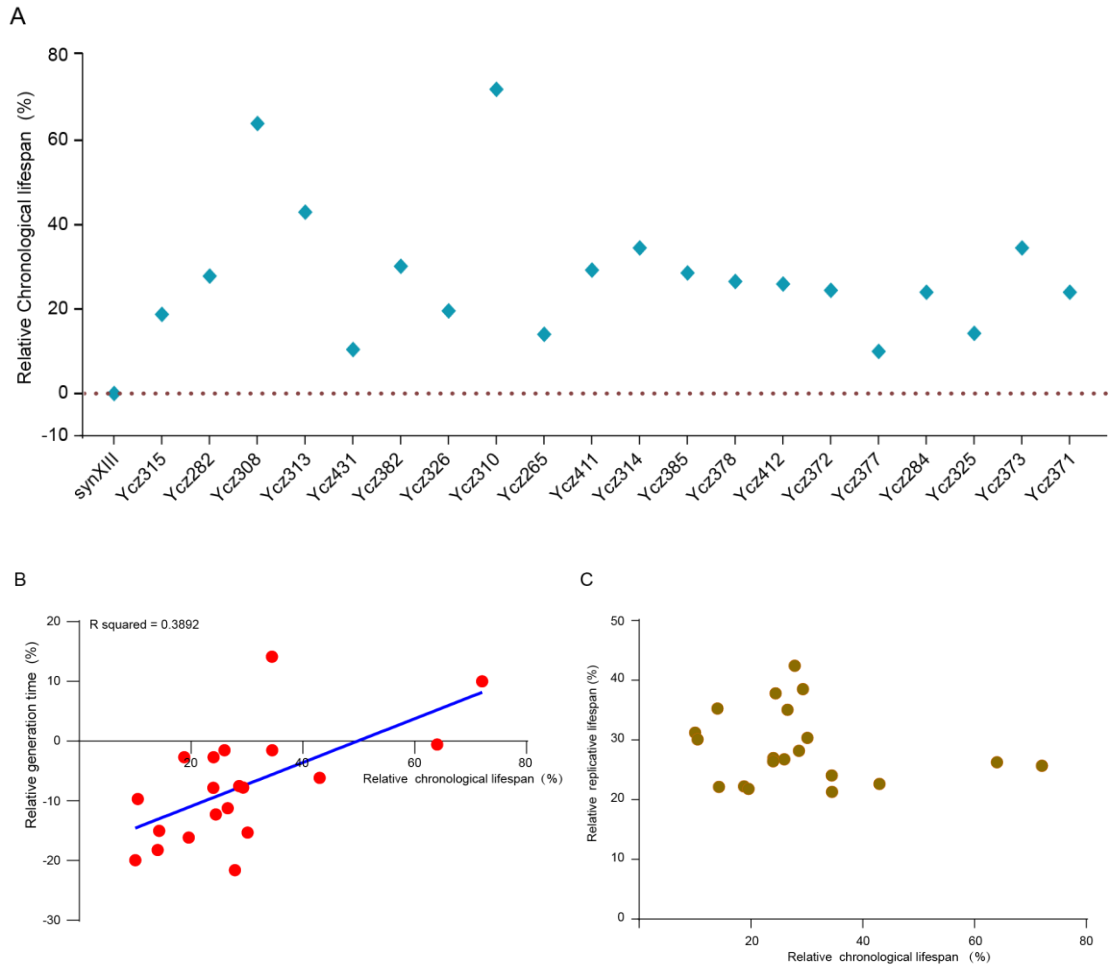
Response 1: We have carefully reviewed and stick to the term "replicative lifespan" to address your concerns and highlighted in blue.

2) For the 21 mutants with increased reproductive lifespan, I appreciate the new data on generation time and the additional discussion. I'd appreciate to also see a plot of the chronological lifespan of the 21 mutants (relative to the original synXIII) for a comprehensive overview of relationships between the generation time, reproductive lifespan, and chronological lifespan of your mutants.

Response 2: To follow your suggestions, we calculated the chronological lifespan (CLS) of the 20 mutants and compared it with that of the parental synXIII strain. The results indicated that all the mutants exhibited a longer chronological lifespan compared to the synXIII (Figure A). This finding suggests that the advantages conferred by our long-lived strains also contribute to an extended chronological lifespan, providing a new avenue for researchers to investigate chronological aging using synthetic yeasts. We have incorporated relevant descriptions and discussions in line 371 - line 375 and Supplementary Figure 10b in line 842 - line 844.

To offer a comprehensive overview of the relationships between generation time, chronological lifespan, and replicative lifespan, we generated scatter plots correlating generation time with chronological lifespan, as well as chronological lifespan with replicative lifespan. The scatter plot indicates a weak negative correlation between generation time and replicative lifespan shows, as shown in Figure B below. A similar trend is observed in Figure C, which also illustrates the weak

relationship between chronological lifespan and replicative lifespan. This suggests that the three may have different biological mechanisms driving them, which could sometimes intersect.



3) Line 294: Still inconsistent: If you write “noncognate UTR” in line 293, then line 294 should be “CDS-noncognate UTR”

Response 3: We have corrected the term to "CDS-noncognate_UTR" to be consistent and highlighted the change in blue in line 293.

Reviewer #2

The authors have largely dismiss my comments. I think the way fitness is measured with simple spot assay inspected by eyes is not quantitative at all. This is the current state of the art when you want to measure yeast growth and fitness:

Response 4. Thank you for your advice. Based on your suggestions, we performed additional growth curve experiments for synthetic strain synXIII together with wild-type strains (BY4741 and BY4742) under different conditions and calculated their doubling times. The results showed that synXIII exhibiting similar or comparable doubling time to the wild-type strains under various conditions.

In addition, to follow your suggestions, we used a high-throughput workflow to measure yeast colony size (PMID: 38020968). We plated ~ 300 colonies for each strain on different conditions respectively at 30 ℃ for 2-3 days. Then, the colonies on the plates were automated identified, photographed, and processed using software that measures colony diameter in terms of millimeters by the Precision Colony Piker (model: PIXL, Singer Instruments, https://www.singerinstruments.com/solution/pixl-precision-microbial-colony-picker/). The relative colony size of each strain was determined by the diameter of individual colonies compared to that of the parental disomic strain. The results indicated synXIII exhibited similar or minor difference compared to wild type strain (BY4741 or BY4742) as presented in Figure B below. We have incorporated relevant descriptions in line 162 and line 467 - line 479, and Supplementary Figure 3 and Supplementary Figure 4 in line 786 - line 801.

Figure A:

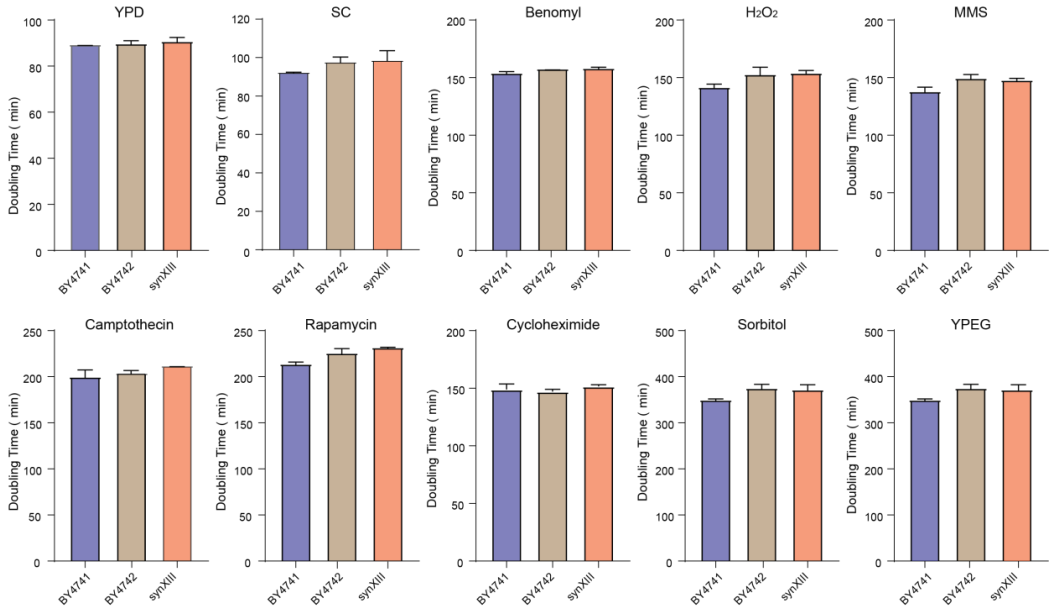
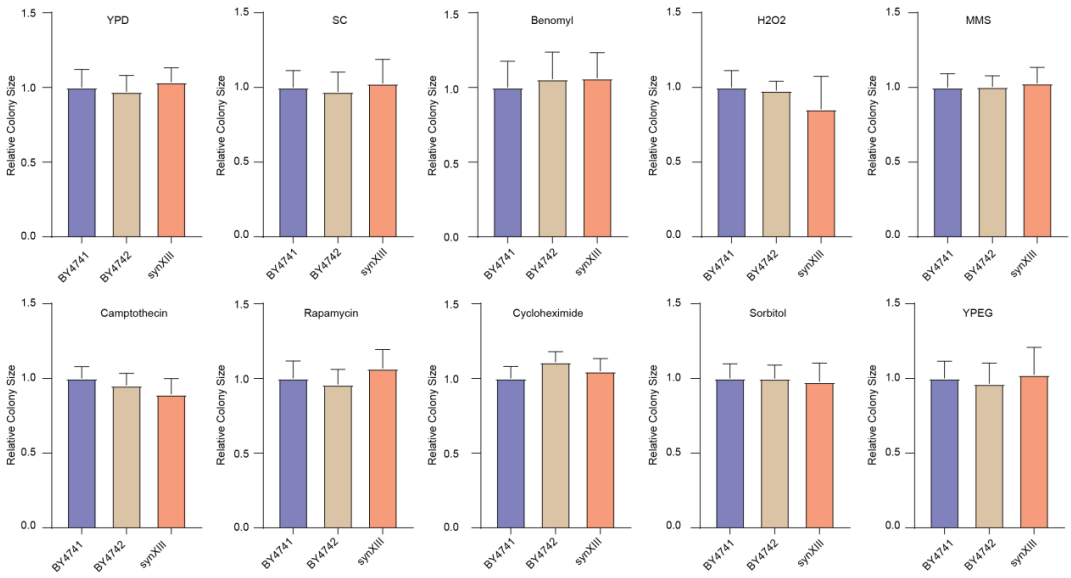


Figure B:

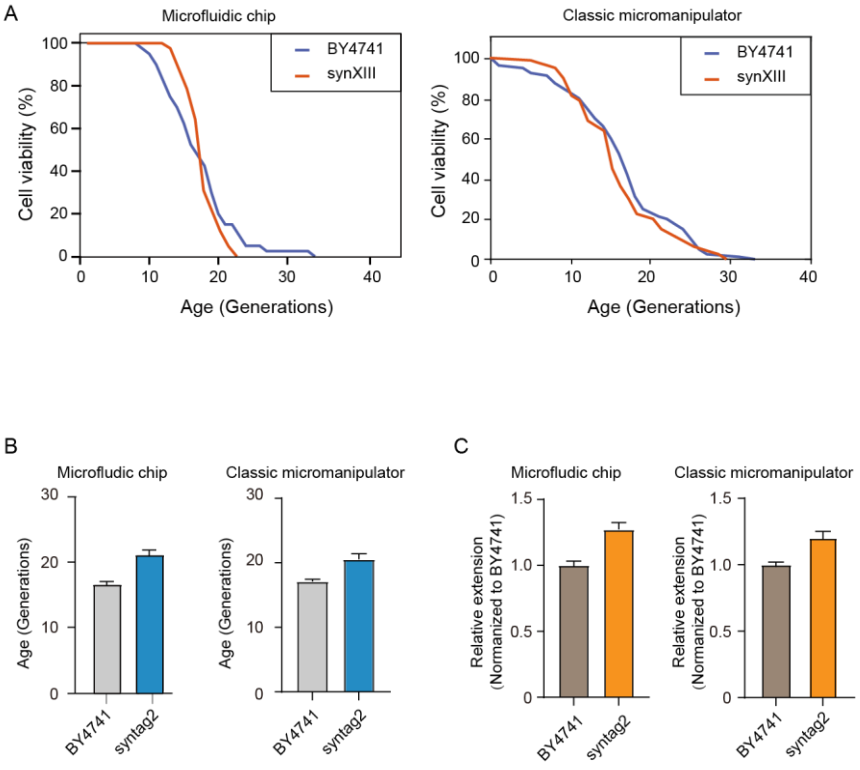


Similarly, validating some of their results with the standard RLS protocol would have make the paper stronger.

Response 5: To follow the reviewer's suggestion, we measured yeast lifespan using the traditional micromanipulator method to validate the RLS data generated by the microfluidic device (PMID: 13666896). We chose two sets of samples for this purpose. First, using the classical micro manipulator approach, we generated the survival curves for the synthetic strain synXIII and the wild-type strain BY4741 and found they exhibited similar lifespans. Specifically, we find the average lifespan for BY4741 and synXIII is 16.44 and 15.9, respectively, as shown in the right lane of panel A (below). Similar survival curves were observed using the microfluidic device as shown in left panel of panel A (below). For the second set of samples, we selected the synonymous mutant long-lived strain syntag2 compared to the wild-type control strain for lifespan validation. The syntag2 exhibited the mean RLS is 19.73, while using microfluidic device, the mean RLS is 20.85 (panel B below). We also compared the extension rate (normalized BY4741 to 1) of syntag2 using two methods. We found that using microfluidic device, the lifespan of syntag2 is 1.27 that of the wild-type BY4741. In contrast, using the classic micromanipulator, it is 1.20 that of the wild-type strain (Figure C). Both microfluidic device and the classic micromanipulator analysis showed that syntag2 exhibited a longer lifespan compared to the wild-type strain.

Therefore, our new results demonstrated that RLS data measured by microfluidic techniques is consistent with the classical micro manipulator approach, highlighting the reliability of our RLS

data presented in the manuscript.



We would like to thank the reviewers for their time and suggestions that helped us improve our work. Below, we provide a point-by-point response (in blue color) to reviewers' comments.

Reviewer #1 (Remarks to the Author):
Thank you for the final revisions. My questions and concerns have been addressed and I find the manuscript mature for publication in Nature Communications.
Response: We thank the reviewer for approval of our study.

Reviewer #2 (Remarks to the Author):
I'm happy with the revised paper and the inclusion of the additional experiments performed (colony size and daughter cells micro manipulation) that confirm the cell growth and RLS findings.
Response: We sincerely appreciate the reviewer for the positive feedback on our article.