nature portfolio

Peer Review File

Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan

Corresponding Author: Dr Mohammad Alfatah

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Summary

Both calorie restriction (CR) and rapamycin (RM) are well-known interventions to extend the healthspan and lifespan across different species, including yeast. In this manuscript, the authors employ RNA-sequencing to analyze the impact of CR, RM, and their combination (CR+RM) on the transcriptome of yeast cells. They uncover distinctive, overlapping, and even contrasting patterns of gene regulation at the transcriptome level, as reflective of the unique and shared effects of CR and RM. They also reveal a synergistic effect of CR+RM on extending the lifespan of post-mitotic cells in yeast. And this effect is also conserved in post-mitotic human cells.

Comments

There are several issues that should be addressed before this manuscript is approved.

1. Fig.1a. The title of this manuscript is “Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan”, whereas it appears that the experimental design and data analysis deviate from the theme. The experimental design shows that only very young cells (both mitotic and post-mitotic) are collected for the RNA-seq. Although the transcriptome profiles of these young cells are different under the treatments of CR and/or RM, it's very weak to unveil their synergistic interaction in prolonging cellular lifespan.

Therefore I recommend the authors to not only analyze the very young cells but also middle-aged and late-aged cells under the treatments of CR and/or RM. Such analysis would be of high interest to the audience. Please refer to the report published recently regarding the early heterogeneity during aging in budding yeast revealed by single-cell RNA-seq for reference (Wang et al, PMID: 36181361).

2. Line 25, Page 6. “A vehicle control group was also included, wherein cells were treated with the DMSO solvent.”

I would like to see an additional control group wherein cells were not treated with DMSO. And the two control groups should be compared as well to exclude the possibility that DMSO itself may also have an effect on the cells.

3. Line 21~23, Page 8. “Among these shared genes, 9 were consistently upregulated, and 41 were consistently downregulated (Figure. 2b), with 49 genes exhibiting divergent expression patterns (Figure. 2c).”

Please list the names of the shared genes under the treatment of CR and plot the gene expression values. Also recommend

the authors to select some of them to do a qPCR. GO analysis of the 49 genes exhibiting divergent expression patterns would also be of interest.

4. Line 5~10, Page 10. “Surprisingly, maltose catabolic genes, including MAL11/12/31/32 and IMA1, were downregulated in RM-treated MiC (Figure. S3b). This suggests that rapamycin may inhibit the utilization of maltose as a carbon source by the cells. Rapamycin's downregulation of maltose metabolism genes could potentially shift the cellular preference even more towards glucose, inhibiting the yeast cells' ability to efficiently utilize maltose.”

I recommend the authors to provide some additional supporting evidence to support this paragraph. It seems to be purely speculative from the RNA-seq data only.

5. Line 19~22, Page 11. “Notably, CR specifically upregulated genes associated with glucose metabolism, whereas rapamycin had the opposite effect, uniquely downregulating these genes (Figures. 4b and 4c; S4a and S4b; Additional File 5).”

The authors may want to explain this paragraph and the paragraph of Line 5~10, Page10 as mentioned above. It seems to contradict with each other.

6. Line 7~9, Page 13. “During the exponential phase, the combined CR+RM treatment uniquely upregulated four genes (VHR2, MLS1, SFC1, SIP18) and downregulated four genes (FIT3, PDC6, FRE5, MND1) (Figure. 6a).”

It’s very interesting that the authors find FIT3 is downregulated under the treatment of CR+RM. FIT3 is a facilitator of iron transport, and is induced upon iron deprivation or mitochondrial DNA loss (Veatch et al., PMID:19563757). We also know the deletion of FIT3 extends the replicative lifespan in yeast (Wang et al., PMID: 36181361). The authors may want to do a qPCR of FIT3 to validate and discuss about this. In addition, I recommend the authors to do a replicative lifespan assay of CR+RM to see if it’s long-lived as well (Fig. 7a/c).

Acceptance

I recommend publication of this work providing the authors address the issues listed above.

Reviewer #2

(Remarks to the Author)
This is a fine manuscript, providing added support to the idea that rapamycin is not simply a mimetic of calorie restriction, engaging distinct cellular responses. The manuscript is well written and logical, and I think will be generally received well by a wide audience.

I think the major issue is a lack of appropriate citations - liver, adipose and similar profiling on rapamycin vs. CR vs. both have been performed almost a decade ago, yet the authors only cite more recent studies rather than the original findings: PMIDs 24304444 and 25075714 for example.

It would also be interesting to know if there are similar effects of rapamycin and CR on the spliceosome as has been observed in worms: PMID 27919065

Reviewer #3

(Remarks to the Author)
Aging is a complex trait. Lifespan is amenable by genetics and environmental factors such as stressors and nutrients. In this manuscript the authors examine gene expression patterns using RNAseq analyses primarily in yeast cells treated with rapamycin (RM), cells grown in reduced levels of glucose compared to the controls (naming of this group is according to the authors calorie restriction-CR) and combination of the drug and restriction combination (RM+CR). The ultimate aim is to identify gene expression signatures that are shared, are opposed or even been unique between treatments that prolong lifespan. The main message of the manuscript is that there are synergistic effects between RM and CR. Additionally, that this knowledge revolutionizes the field and will provide a breakthrough for aging and age-related diseases.

Unfortunately, in the current form the manuscript is not acceptable for publication. I believe that there are serious omissions/flaws that need to be addressed . In addition, the efforts are concentrated on the level of transcriptome without assessing anything on a post-transcriptional level or provide validation for a few chosen candidates that are novel targets of RM+CR only and how they affect lifespan and metabolism of yeast and human cells. The manuscript, therefore, requires rethinking
even if chosen to be submitted to a more specialized journal.

Comments and suggestions:
1. The authors claim that the use of the yeast model offers opportunities to examine biological questions through the design and conduct of highly controlled experiments. This is very true; nevertheless, the experimental design is very poor while they should provide evidence of control and the state of cells at the exact timepoints used: The authors dilute cultures at 0.2 OD and then examine gene expression in the different conditions (control, RM, CR, RM+CR) after 5 hrs (mitotic) and after 72 hrs (postmitotic). However, they should have provided growth patterns in all conditions as RM, CR, RM+CR will cause growth

delays. Although 5hrs timepoints might be comparable, the aformentioned delays will have effects on the later timepoints as cells of the different groups might enter stationary phase at very different times. The authors should provide growth curves and use appropriate timepoints for the different conditions examined that are comparable (e.g. 1, 2, 3, 4 days etc following entrance to stationary phase). I find this to be a major problem of the manuscript as all subsequent data are based on this flawed scheme.

2. The synergy between RM and CR is not an original idea. Synergy in fly lifespan between the two is previously reported (PMID: 20074526, 35681084). Differential effects of the two have been reported in progeroid mice (PMID: 33484480), while effects of CR+RM combinations in transcriptomes and metabolomes have been examined in various contexts (e.g. PMID: 24304444).

3. The effects of CR and RM treatments in both yeast and human cells must be validated using markers for translational status, autophagy etc. How does a combination of CR+RM differ compared to CR or RM only in such markers?

4. The authors should also validate some findings to solidify their data and their claims. For example:
Sugar metabolism – there seem to be differences in sugar metabolism between the treatments, how can this be confirmed further? There are metabolism assays available on the market that can help confirm these changes which have not been employed? Further experiments should have been done to elucidate this further.

5. Following the previous point: the authors could accompany their study with metabolome or to chose a few candidate genes that they think are central or interesting for being regulated by RM+CR. The authors can then validate whether these genes are required for RM+CR-dependent lifespan extensions observed.

6. Lifespans should be conducted using CFUs and provide lifespan curves that are widely used in the field. While spot assays provide snapshots for chosen days, lifespan curves provide all the information and viability dynamics.

7. The HEK293 post-mitotic cell assay used should be described in better detail and provide more justification for its use and how it relates to normal cellular ageing and lifespan. HEK293 cells are 'pushed' into a post-mitotic cell condition by addition of D10 medium (which is freezing medium – contains 10% DMSO in FBS). A continuous viability assay similar to the yeast chronological lifespan assay should be used to give insights on the dynamics of cell viability over days and the effects of RM, CR and RM+CR.

8. The figures require re-design as many labels are very difficult to read. For example the PCA in Fig.1C, the labels and colors of the different groups/conditions are almost impossible to see. Figures throughout small labels, very small fonts. Supplementary figure S6 – overlapping labels.

9. Authors should make sure that all sequencing data are deposited in appropriate databases and available to the readers and the community (they should be available to reviewers and editors).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Remarks to the Author:
I have read through the revised manuscript and rebuttal letter from the authors. And I am concerned with some important issue before approving the publication of this paper.

As I emphasized in the previous review report, while the title of this manuscript is “Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan”, the experimental design and data analysis deviate from this theme. Specifically, the experimental design only collects very young cells (5 hr for mitotic and 72 hr for post-mitotic, respectively) for RNA-seq, which I belive is a major flaw of this paper. Only transcriptome profiles of these very young cells under CR and/or RM are neither adequate nor sufficient to unveil their synergistic interaction in prolonging cellular lifespan (Aging is a complicated biological proces, and not just one time point).

So I recommended the authors to not only analyze the very young cells but also middle-aged and late-aged cells under CR and/or RM. Such analysis during the aging process (instead of just one time point at very young age), would be very important and valuable in deciphering the synergistic interaction in prolonging cellular lifespan. However, it seems that the authors haven’t realised the importance of this issue and haven’t done such experiments and analysis as suggested.

This was also mentioned by the Reviewer #3: “The experimental design is very poor... the authors should provide growth curves and use appropriate timepoints for the different conditions examined that are comparable (e.g. 1, 2, 3, 4 days etc following entrance to stationary phase). I find this to be a major problem of the manuscript as all subsequent data are based on this flawed scheme.”

In general, I don't see too much strong evidence supporting that systematic transcriptomics analysis of very young yeast cells treated with calorie restriction and rapamycin can unveil their synergistic interaction in prolonging cellular lifespan. And I am not persuaded that this manuscript represents a sufficient advance in our understanding of the synergistic interaction between calorie restriction and rapamycin in prolonging cellular lifespan to justify publication in Communications Biology. I suggest the authors change the title of this manuscript and submit to a more specialized journal.

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed the concerns.

Reviewer #3

(Remarks to the Author)
The authors' efforts towards improving the manuscript are greatly appreciated.

Please see below: numbers refer to the original review points.

1. The point is addressed. Many thanks for changing the figure to growth curve. It helps the readers to realise that the treatments do not induce a lag phase and both treated and untreated cells enter stationary phase in the same time period.

2. The authors acknowledge that the synergy of R and CR in lifespan is not a novel phenomenon. Therefore, the abstract key statement 'Most notably, our findings reveal a synergistic effect of CR+RM in extending the lifespan of postmitotic cells, a result validated in both yeast and human cells' should be amended accordingly. My original comment relates on phrasing results of the study as novel (our findings…reveal).

3. I understand that the focal point is a comparison of RNAseq data from R and CR conditions. Transcriptome-derived GO enrichments do not address the original point of the reviewer. The point becomes even more important as the provided growth curves do not show any growth phenotype following R and CR conditions (Fig.1b). Is there any mTOR-related marker change in these treatments? The question remains.
While gene transcripts change, these changes do not necessarily translate into changes of protein levels too.

4. This point relates to point 3. And, to the fact that mRNA changes do not necessarily translate into changes of protein levels. The authors decided to validate RNA levels through RT-PCRs instead of a single western blot of HXK1 in one of the conditions where the corresponding gene levels are changed? The question remains.
The authors claim in the abstract that: 'This research … presents potential strategies for enhancing healthspan and delaying the onset of age-related diseases. These findings have the potential to revolutionize our approach to implementing these interventions'. This reviewer finds this central statement very strong without any validation of RNASeq data.

5. The point relates to point 4. I understand and agree with the authors that comprehensive analyses are not the scope of the paper. That's why I suggested only validating a few candidates.

6. The derived graph plot provided by the authors is acceptable. The authors have addressed the point.

7. The authors have addressed the point.

8. I appreciate the effort of authors to improve the figures but the point remains. Fonts can be very small and difficult to read throughout. For example, see labels/fonts of categories in Figure 2e.

9. Many thanks for the data deposition, I anm sure this will be checked by the journal too.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have done text changes that have been recommended.

However, they have ignored further suggestions and comments using the phrase 'beyond the scope of the manuscript' although a justification for the given points has been provided by this reviewer.

Specifically:

Point 3: not addressed as 'beyond the scope'. Justification for this ask has been provided. The ask was for validation of 1-2 results only to solidify the claims from the RNA-seq results, extensive discussion and development of scenarios.

Point 4: while the authors made a text change in the abstract to remove very strong statements, they have ignored the first

part of the point. They have decided to provide RT-PCR data in the previous round of revision, instead of a couple simple western blots for the proteins of interest. This is very surprising. I have raised the point once again and it is still not addressed.

Point 5: not addressed. I agree with the authors that a comprehensive protein-level analysis is not required for the manuscript. I have asked only for a couple of validations related e.g. to HXK1. I've never agreed with the authors on point 5.

Point 8: while high-quality images are provided, the fonts and arrangements of figures still need attention. They are very populated and busy with tiny fonts.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have now provided an example of protein-level evidence that supports their claims from the RNA-seq data analyses. They have used a tagged version of Glk1 and have provided western blots with various concentrations of rapamycin. They have also quantified the obtained signals and normalised with actin. Their quantiifcations are accompanied with appropriate statistical tests.

The authors have also answered to all other raised points.

I am, therefore, happy with these revisions and believe that the manuscript is suitable for publication.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Summary

Both calorie restriction (CR) and rapamycin (RM) are well-known interventions to extend the healthspan and lifespan across different species, including yeast. In this manuscript, the authors employ RNA-sequencing to analyze the impact of CR, RM, and their combination (CR+RM) on the transcriptome of yeast cells. They uncover distinctive, overlapping, and even contrasting patterns of gene regulation at the transcriptome level, as reflective of the unique and shared effects of CR and RM. They also reveal a synergistic effect of CR+RM on extending the lifespan of post-mitotic cells in yeast. And this effect is also conserved in post-mitotic human cells.

Author Response to Reviewer #1:

Thank you for reviewing and summarizing our findings.

Comments

There are several issues that should be addressed before this manuscript is approved.

1. Fig.1a. The title of this manuscript is “Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan”, whereas it appears that the experimental design and data analysis deviate from the theme. The experimental design shows that only very young cells (both mitotic and post-mitotic) are collected for the RNA-seq. Although the transcriptome profiles of these young cells are different under the treatments of CR and/or RM, it's very weak to unveil their synergistic interaction in prolonging cellular lifespan.

Therefore I recommend the authors to not only analyze the very young cells but also middle-aged and late-aged cells under the treatments of CR and/or RM. Such analysis would be of high interest to the audience. Please refer to the report published recently regarding the early heterogeneity during aging in budding yeast revealed by single-cell RNA-seq for reference (Wang et al, PMID: 36181361).

Author Response to Reviewer #1:

Thank you for your insightful comments and the reference to the study by Wang et al. (PMID: 36181361). We appreciate your consideration and would like to provide additional clarification on our experimental design. Wang et al. conducted RNA-seq at three time points (2h, 16h, and 32h) and identified heterogeneity between the 16hr and 32hr time points (PMID: 36181361; Figure 2a).

We acknowledge the importance of the study by Wang et al., which focused on early heterogeneity during aging in budding yeast using single-cell RNA-seq. In contrast to their study, our investigation does not consider heterogeneity, addressing the primary concern raised by Reviewer 1. Our study specifically conducted transcriptomics at 5 hours for mitotic cells.

Furthermore, we want to highlight that our study distinctly focuses on the transcriptomics of mitotic (dividing) and postmitotic (non-dividing) cells.

As mentioned in the introduction (6th paragraph, page 5), yeast cells undergo a transition from fermentative to respiratory metabolism, known as the diauxic shift, followed by the onset of the stationary phase. This transition, associated with heterogeneity, is not crucial for our investigation.

Our study centers on analyzing transcriptomics for the exponential and stationary phases of yeast culture, representing optimal conditions for studying mitotic and postmitotic cells. For postmitotic cells, we chose 72 hours, a time point where all cells cease dividing and completely enter the post-mitotic stage (Figure 1b), recommended for analyzing the lifespan of postmitotic cells (ref 45; PMID: 22768836).

Our study analyzed transcriptomics of mitotic and postmitotic for four conditions (control, CR, RM, and CR+RM), rather than young cells vs old cells (mitotic and postmitotic). In contrast, the study by Wang et al. focuses on a single condition at three time points (2 hours, 16 hours, and 32 hours), which is not specifically centered on the stationary phase relevant to postmitotic cellular lifespan.

Our transcriptomics data clearly reveals the synergistic interaction of CR+RM in both mitotic and postmitotic cells (Figure 6 and New Additional Files 6, 6a and 6b). We have further validated transcriptomics results into the phenotypic synergetic lifespan extension of postmitotic cells (chronological lifespan) (Figure 7 and New Figure S11).

However, we have not shown the extension of mitotic lifespan (replicative lifespan) as we don't have a platform to test this replicative lifespan. We are open to changing our manuscript title from "Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan" to "Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging the lifespan of postmitotic cells."

We appreciate your diligence in reviewing our manuscript, and we are committed to addressing any further concerns to enhance the clarity of our study.

2. Line 25, Page 6. “A vehicle control group was also included, wherein cells were treated with the DMSO solvent.”

I would like to see an additional control group wherein cells were not treated with DMSO. And the two control groups should be compared as well to exclude the possibility that DMSO itself may also have an effect on the cells.

Author Response to Reviewer #1:

We appreciate the opportunity to address your concern, and we would like to provide clarification regarding the rationale behind our experimental design.

The vehicle control (DMSO solvent) was chosen because the chemical Rapamycin used in our experiment was prepared in a DMSO. Consequently, both the control group and CR

conditions were treated with the same amount of DMSO to ensure consistency in the experimental conditions.

In our RNA-sequencing analysis, we employed rapamycin-treated cells as the treatment group, comparing them to the control group treated with the DMSO. This approach allows us to obtain differentially expressed genes in relative terms, representing changes in gene expression resulting from Rapamycin alone. The DMSO effect that occurred in both the treatment and control groups was omitted through this comparative analysis.

Reviwer 1 proposed control group comparisons, such as Control group (untreated) vs Control group (DMSO treated), would primarily represent the DMSO effect on cells. On the other hand, Control (untreated) vs Treatment group (Rapamycin treated) would represent the combined effect of DMSO and Rapamycin on cells, not the Rapamycin effect alone. Therefore, we believe that additional control group comparisons may not provide significant additional insights in this specific context.

For more detailed information, we included the relevant details in the "Differential Gene Expression Analysis" section of the Methods, where we elaborated on the specific steps taken to control for potential confounding effects and explain the rationale behind our chosen comparisons.

We hope this explanation clarifies our experimental design choices and underscores our confidence in the validity of our comparative analyses.

3. Line 21~23, Page 8. “Among these shared genes, 9 were consistently upregulated, and 41 were consistently downregulated (Figure. 2b), with 49 genes exhibiting divergent expression patterns (Figure. 2c).”

Please list the names of the shared genes under the treatment of CR and plot the gene expression values. Also recommend the authors to select some of them to do a qPCR. GO analysis of the 49 genes exhibiting divergent expression patterns would also be of interest.

Author Response to Reviewer #1:

Thank you for your suggestion. The list of shared genes under the Calorie Restriction (CR) treatment is available in "Additional File 2." Further we have included a more accessible presentation of this information in the main manuscript and provided “Additional File 2a” for 9 gene list and “Additional File 2b” for 41 gene and “Additional File 2c” for 49 gene list. Additionally, we have conducted qRT-PCR for selected genes from both upregulated (Figure. S2b) and downregulated list (Figure.S2c) and perform Gene Ontology (GO) analysis on the 49 genes with divergent expression patterns (Figure.S2d), further strengthening the validity and interpretation of our results.

4. Line 5~10, Page 10. “Surprisingly, maltose catabolic genes, including MAL11/12/31/32 and IMA1, were downregulated in RM-treated MiC (Figure. S3b). This suggests that rapamycin may inhibit the utilization of maltose as a carbon source by the cells. Rapamycin's downregulation of maltose metabolism genes could potentially shift the cellular preference

even more towards glucose, inhibiting the yeast cells' ability to efficiently utilize maltose."

I recommend the authors to provide some additional supporting evidence to support this paragraph. It seems to be purely speculative from the RNA-seq data only.

Author Response to Reviewer #1:

Thank you for your valuable feedback and suggestion. We appreciate your concern regarding the speculative nature of our statement on rapamycin's potential inhibition of maltose utilization based solely on RNA-seq data.

To address this, we have identified additional supporting evidence. Specifically, we observed that rapamycin downregulates several glucose metabolism genes, including those involved in maltose catabolism (MAL12/MAL32/IMA1), as evidenced in Additional File 3.

This aligns with our broader findings that rapamycin induces a conserved transcriptomic signature in glucose metabolism, as recently reviewed (PMID: 33599151).

To further substantiate our claim, we have cited PMID: 33599151 as reference 59 in the revised manuscript, validated the RNA-seq results through qRT-PCR for glycolytic genes (HXK1 and GLK1) and maltose catabolic genes MAL11/12/31/32 and IMA1 (Figure. S3e), and supplement our analysis with Gene Ontology (GO) results for the 134 consistently downregulated genes in both mitotic and postmitotic cells (Additional File 3b; Figure. S3b).

We appreciate your insightful suggestions and are committed to enhancing the robustness and validity of our manuscript.

5. Line 19~22, Page 11. "Notably, CR specifically upregulated genes associated with glucose metabolism, whereas rapamycin had the opposite effect, uniquely downregulating these genes (Figures. 4b and 4c; S4a and S4b; Additional File 5)."

The authors may want to explain this paragraph and the paragraph of Line 5~10, Page10 as mentioned above. It seems to contradict with each other.

Author Response to Reviewer #1:

Thank you for bringing attention to the potential contradiction in our interpretation. We appreciate your careful review, and you are correct in identifying a misinterpretation. We acknowledge the need for clarification and will promptly revise the mentioned paragraphs.

The revised version has accurately reflect the downregulation signature of glucose metabolism including maltose by rapamycin, aligning with our RNA-seq data with PMID: 33599151 as reference 59 citation. Your feedback is invaluable, and we are committed to improving the consistency and clarity of our manuscript.

6. Line 7~9, Page 13. "During the exponential phase, the combined CR+RM treatment uniquely upregulated four genes (VHR2, MLS1, SFC1, SIP18) and downregulated four

genes (FIT3, PDC6, FRE5, MND1) (Figure. 6a)."

It's very interesting that the authors find FIT3 is downregulated under the treatment of CR+RM. FIT3 is a facilitator of iron transport, and is induced upon iron deprivation or mitochondrial DNA loss (Veatch et al., PMID:19563757). We also know the deletion of FIT3 extends the replicative lifespan in yeast (Wang et al., PMID: 36181361). The authors may want to do a qPCR of FIT3 to validate and discuss about this. In addition, I recommend the authors to do a replicative lifespan assay of CR+RM to see if it's long-lived as well (Fig. 7a/c).

Author Response to Reviewer #1:

Thank you for your valuable suggestions. We have validated the expression of the FIT3, FRE5, and FIT2 genes using qRT-PCR (see Figure S9a) and included a discussion with a citation (Wang et al., PMID: 36181361, reference 60) of the results. Unfortunately, as mentioned earlier, we are unable to conduct the suggested replicative lifespan assay due to the unavailability of a suitable platform.

Acceptance
I recommend publication of this work providing the authors address the issues listed above.

Author Response to Reviewer #1:

Thank you for your positive recommendation. We have addressed the points that you have highlighted above to enhance the manuscript's quality.

Reviewer #2 (Remarks to the Author):

This is a fine manuscript, providing added support to the idea that rapamycin is not simply a mimetic of calorie restriction, engaging distinct cellular responses. The manuscript is well written and logical, and I think will be generally received well by a wide audience.

I think the major issue is a lack of appropriate citations - liver, adipose and similar profiling on rapamycin vs. CR vs. both have been performed almost a decade ago, yet the authors only cite more recent studies rather than the original findings: PMIDs 24304444 and 25075714 for example.

It would also be interesting to know if there are similar effects of rapamycin and CR on the spliceosome as has been observed in worms: PMID 27919065

Author Response to Reviewer #2:

Thank you for your positive feedback on our manuscript and for your constructive comments. We appreciate your suggestion to include appropriate citations and have incorporated the original findings (PMIDs 24304444 and 25075714) into our revised manuscript as references 65 and 66, respectively.

Furthermore, we value your recommendation to explore the potential effects of rapamycin and calorie restriction (CR) on the spliceosome. We have thoroughly analyzed our data to address this aspect in the results section "Combined Calorie Restriction and Rapamycin-Induced Gene Expression Profiles in MiC and PoMiC." We have included relevant findings (Figures S6, S7, and S8) and cited PMID 27919065 as reference 59 in our revised manuscript.

Reviewer #3 (Remarks to the Author):

Aging is a complex trait. Lifespan is amenable by genetics and environmental factors such as stressors and nutrients. In this manuscript the authors examine gene expression patterns using RNAseq analyses primarily in yeast cells treated with rapamycin (RM), cells grown in reduced levels of glucose compared to the controls (naming of this group is according to the authors calorie restriction-CR) and combination of the drug and restriction combination (RM+CR). The ultimate aim is to identify gene expression signatures that are shared, are opposed or even been unique between treatments that prolong lifespan. The main message of the manuscript is that there are synergistic effects between RM and CR. Additionally, that this knowledge revolutionizes the field and will provide a breakthrough for aging and age-related diseases.

Unfortunately, in the current form the manuscript is not acceptable for publication. I believe that there are serious omissions/flaws that need to be addressed . In addition, the efforts are concentrated on the level of transcriptome without assessing anything on a post-transcriptional level or provide validation for a few chosen candidates that are novel targets of RM+CR only and how they affect lifespan and metabolism of yeast and human cells. The manuscript, therefore, requires rethinking even if chosen to be submitted to a more specialized journal.

Author Response to Reviewer #3:

Thank you for reviewing and summarizing our findings.

Comments and suggestions:

1. The authors claim that the use of the yeast model offers opportunities to examine biological questions through the design and conduct of highly controlled experiments. This is very true; nevertheless, the experimental design is very poor while they should provide evidence of control and the state of cells at the exact timepoints used: The authors dilute cultures at 0.2 OD and then examine gene expression in the different conditions (control, RM, CR, RM+CR) after 5 hrs (mitotic) and after 72 hrs (postmitotic). However, they should have provided growth patterns in all conditions as RM, CR, RM+CR will cause growth delays. Although 5hrs timepoints might be comparable, the aforementioned delays will have effects on the later timepoints as cells of the different groups might enter stationary phase at very different times. The authors should provide growth curves and use appropriate timepoints for the different conditions examined that are comparable (e.g. 1, 2, 3, 4 days etc following entrance to stationary phase). I find this to be a major problem of the manuscript as all subsequent data are based on this flawed scheme.

Author Response to Reviewer #3:

We appreciate your attention to the experimental design and your comments. Growth patterns for all conditions are clearly depicted in Figure 1b, presented in a bar graph format. However, we have change growth patterns (bar graph) into growth curves in our revised manuscript.

It's worth noting that our experimental design includes thorough control experiments, and the growth analysis spans from 5 hours to 72 hours. As evident in the result (Figure 1b), cells reach saturation and halt division after 24 hours, entering the stationary phase. To ensure clarity on the postmitotic phase, we specifically selected the 72-hour timepoint, aligning with the recommended start of chronological lifespan (ref 45; PMID: 22768836).

2. The synergy between RM and CR is not an original idea. Synergy in fly lifespan between the two is previously reported (PMID: 20074526, 35681084). Differential effects of the two have been reported in progeroid mice (PMID: 33484480), while effects of CR+RM combinations in transcriptomes and metabolomes have been examined in various contexts (e.g. PMID: 24304444).

Author Response to Reviewer #3:

Thank you for pointing out these relevant studies on the synergy between Rapamycin and Calorie Restriction. We acknowledge the existing literature and appreciate your insight. Our study uniquely contributes by providing a systematic transcriptomics analysis in mitotic and postmitotic cells, offering valuable insights into the specific cellular aging.

3. The effects of CR and RM treatments in both yeast and human cells must be validated using markers for translational status, autophagy etc. How does a combination of CR+RM differ compared to CR or RM only in such markers?

Author Response to Reviewer #3:

We appreciate your thoughtful suggestion to validate the effects of Calorie Restriction (CR) and Rapamycin (RM) treatments in yeast and human cells using markers for translational status, autophagy, etc. Our current study, as outlined in the manuscript, focuses on comparing the transcriptomics of CR, RM, and the combination CR+RM under mitotic and postmitotic conditions.

We have provided an extensive dataset encompassing four conditions (Control, CR, RM, and CR+RM) for mitotic and postmitotic state. The study has identified unique gene signatures for CR and RM (Figure 6d; Additional File 6), supporting previous findings, and has revealed distinct signatures for CR+RM under both mitotic and postmitotic conditions. We have also validated our synergy results for postmitotic cellular lifespan beyond transcriptional analysis (Figures 7a-d and S11a-d).

While conducting experiments to test markers such as translational status and autophagy, among many other aging hallmarks, for four conditions in both mitotic and postmitotic cells is beyond the scope of the current study. However, we have address your suggestion by including the Biological Process (GO) analysis for the uniquely upregulated (114) and

downregulated (78) genes associated with CR+RM (Figures S10a and S10b; Additional File 6a and Additional File 6b).

4. The authors should also validate some findings to solidify their data and their claims. For example:
Sugar metabolism – there seem to be differences in sugar metabolism between the treatments, how can this be confirmed further? There are metabolism assays available on the market that can help confirm these changes which have not been employed? Further experiments should have been done to elucidate this further.

Author Response to Reviewer #3:

Thank you for your feedback. To support our findings on sugar metabolism, we have included PMID: 33599151 as reference 59 in the revised manuscript, demonstrating rapamycin's impact on glucose metabolism genes, consistent with our observations. While we appreciate the suggestion for metabolism assays, performing them for four conditions in both mitotic and postmitotic cells is beyond the scope of our study. However, we have validated our results by through qRT-PCR for glycolytic genes (HXK1 and GLK1) and maltose catabolic genes MAL11/12/31/32 and IMA1 (Figure. S3e), and supplement our analysis with Gene Ontology (GO) results for the 134 consistently downregulated genes in both mitotic and postmitotic cells (Figure. S3b; Additional File 3b).

5. Following the previous point: the authors could accompany their study with metabolome or to chose a few candidate genes that they think are central or interesting for being regulated by RM+CR. The authors can then validate whether these genes are required for RM+CR-dependent lifespan extensions observed.

Author Response to Reviewer #3:

Thank you for your insightful comment. In response, we want to highlight that our study identified 114 upregulated genes (Additional File 6a) and 78 downregulated genes (Additional File 6a) in postmitotic cells under CR+RM treatment. While we acknowledge the importance of investigating candidate genes, we also note that CR+RM may influence a complex biological process controlled by a group of genes rather than a single gene.

Analyzing each deletion strain and testing their dependency on CR+RM lifespan extension could be a comprehensive project on its own, potentially warranting a separate manuscript to fully explore the intricacies of the synergistic effect.

6. Lifespans should be conducted using CFUs and provide lifespan curves that are widely used in the field. While spot assays provide snapshots for chosen days, lifespan curves provide all the information and viability dynamics.

Author Response to Reviewer #3:

We appreciate your feedback and the suggestion to conduct lifespan assessments using colony-forming units (CFUs) and provide lifespan curves. In response, we would like to highlight that our postmitotic cellular lifespan assessments were conducted using standard outgrowth methods (PMID: 16418483 (ref 31) and PMID: 35705837 (ref 61)). This method allowed for the examination of lifespan in 96-well plates for multiple conditions simultaneously, reducing variability compared to CFU-based analysis.

It is crucial to emphasize that our testing methodology encompassed both outgrowth in liquid medium (Figure. 7a) and spot assays (Figure. 7c). This information is explicitly outlined in the method section titled "Analysis of postmitotic cellular lifespan of yeast during chronological aging." Additionally, cell survival was quantified at various chronological aging time points, as illustrated in Figure 7b. To address your suggestion, we have provided lifespan graph plot derived from the Figure. 7b quantified data (New figure S11a-d), illustrating the lifespan trajectories across different conditions during chronological aging.

7. The HEK293 post-mitotic cell assay used should be described in better detail and provide more justification for its use and how it relates to normal cellular ageing and lifespan. HEK293 cells are 'pushed' into a post-mitotic cell condition by addition of D10 medium (which is freezing medium – contains 10% DMSO in FBS). A continuous viability assay similar to the yeast chronological lifespan assay should be used to give insights on the dynamics of cell viability over days and the effects of RM, CR and RM+CR.

Author Response to Reviewer #3:

Thank you for your comments. Firstly, we would like to clarify that D10 medium refers to High-glucose DMEM supplemented with 10% FBS and 1% Pen/Strep. Contrary to the information provided, no DMSO is added to the medium. This common cell culture medium is essential for maintaining optimal conditions for HEK293 cell growth.

Secondly, we acknowledge your recommendation for a continuous viability assay similar to the yeast chronological lifespan assay. We have used a method that we recently published, utilizing a propidium iodide (PI) fluorescent-based approach for determining lifespan in human cells (PMID: 38254217), and we have now appropriately cited this reference as 62 in the revised manuscript.

8. The figures require re-design as many labels are very difficult to read. For example the PCA in Fig.1C, the labels and colors of the different groups/conditions are almost impossible to see. Figures throughout small labels, very small fonts. Supplementary figure S6 – overlapping labels.

Author Response to Reviewer #3:

Thank you for highlighting concerns about figure legibility. We acknowledge the issue. We improved figures, label and font visibility in the revised manuscript.

9. Authors should make sure that all sequencing data are deposited in appropriate

databases and available to the readers and the community (they should be available to reviewers and editors).

Author Response to Reviewer #3:

Thank you for highlighting the importance of data accessibility. We have provided Data availability section in the revised manuscript and included the information of sequencing data deposited in SRA under accession codes SAMN39059417, SAMN39059418, SAMN39059419, SAMN39059420, SAMN39059421, SAMN39059422, SAMN39059423, SAMN39059424, SAMN39059425, SAMN39059426, SAMN39059427, SAMN39059428, SAMN39059429, SAMN39059430, SAMN39059431, SAMN39059432

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Remarks to the Author:

I have read through the revised manuscript and rebuttal letter from the authors. And I am concerned with some important issue before approving the publication of this paper.

As I emphasized in the previous review report, while the title of this manuscript is “Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan”, the experimental design and data analysis deviate from this theme. Specifically, the experimental design only collects very young cells (5 hr for mitotic and 72 hr for post-mitotic, respectively) for RNA-seq, which I belive is a major flaw of this paper. Only transcriptome profiles of these very young cells under CR and/or RM are neither adequate nor sufficient to unveil their synergistic interaction in prolonging cellular lifespan (Aging is a complicated biological proces, and not just one time point).

So I recommended the authors to not only analyze the very young cells but also middle-aged and late-aged cells under CR and/or RM. Such analysis during the aging process (instead of just one time point at very young age), would be very important and valuable in deciphering the synergistic interaction in prolonging cellular lifespan. However, it seems that the authors haven’t realised the importance of this issue and haven’t done such experiments and analysis as suggested.

In the previous revision, we had already addressed the major concern about heterogeneity raised by Reviewer 1, based on the study published in Aging Cell, 2022 (Wang et al., PMID: 36181361), led by groups including Xiannian Zhang, Yi Zhang, and Yanyi Huang. However, Reviewer 1 has suggested further analysis of transcriptomics at time points following the entrance to the stationary phase.

We believe this suggestion is not ideal because cells gradually die during chronological aging (Figure 7 A-C). Therefore, transcriptomic analysis of a mixed population of viable and dead cells would be irrelevant and infeasible under the current study conditions: (1) Control, (2) CR, (3) RM, and (4) CR+RM.

This was also mentioned by the Reviewer #3: “The experimental design is very poor... the authors should provide growth curves and use appropriate timepoints for the different conditions examined that are comparable (e.g. 1, 2, 3, 4 days etc following entrance to stationary phase). I find this to be a major problem of the manuscript as all subsequent data are based on this flawed scheme.”

In our previous revision, we explained our rationale for choosing 5 hours and 72 hours as the ideal time points for studying mitotic and postmitotic cells. Additionally, we have provided the growth curve data requested by Reviewer 3, which addresses the concerns raised by Reviewer 1.

In general, I don’t see too much strong evidence supporting that systematic transcriptomics analysis of very young yeast cells treated with calorie restriction and rapamycin can unveil their synergistic interaction in prolonging cellular lifespan.

And I am not persuaded that this manuscript represents a sufficient advance in our understanding of the synergistic interaction between calorie restriction and rapamycin in prolonging cellular lifespan to justify publication in Communications Biology. I suggest the authors change the title of this manuscript and submit to a more specialized journal.

Our transcriptomics data clearly reveals the synergistic interaction of CR+RM in both mitotic and postmitotic cells (Figure 6 and Additional Files 6, 6a, and 6b). This information is detailed in the text under the section "Comparing combined calorie restriction and rapamycin-induced gene expression profiles with individual interventions in MiC and PoMiC." Furthermore, we have validated our transcriptomics results by demonstrating the phenotypic synergistic lifespan extension of postmitotic cells (chronological lifespan) (Figure 7 and New Figure S11).

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed the concerns.

Thank you for your positive feedback and for acknowledging that we have satisfactorily addressed your concerns.

Reviewer #3 (Remarks to the Author):

The authors' efforts towards improving the manuscript are greatly appreciated.

Thank you for your kind words and for appreciating our efforts to improve the manuscript.

Please see below: numbers refer to the original review points.

1. The point is addressed. Many thanks for changing the figure to growth curve. It helps the readers to realise that the treatments do not induce a lag phase and both treated and untreated cells enter stationary phase in the same time period.

Thank you for acknowledging the changes to the figure. We are glad the revised growth curve clarifies the treatment effects.

2. The authors acknowledge that the synergy of R and CR in lifespan is not a novel phenomenon. Therefore, the abstract key statement ‘Most notably, our findings reveal a synergistic effect of CR+RM in extending the lifespan of postmitotic cells, a result validated in both yeast and human cells’ should be amended accordingly. My original comment relates on phrasing results of the study as novel (our findings…reveal).

Thank you for your suggestions. We have amended the abstract accordingly.

3. I understand that the focal point is a comparison of RNAseq data from R and CR conditions. Transcriptome-derived GO enrichments do not address the original point of the reviewer. The point becomes even more important as the provided growth curves do not show any growth

phenotype following R and CR conditions (Fig.1b). Is there any mTOR-related marker change in these treatments? The question remains.

While gene transcripts change, these changes do not necessarily translate into changes of protein levels too.

We recognize the importance of rigorous validation. However, conducting protein-level validation is beyond the scope of our current study.

4. This point relates to point 3. And, to the fact that mRNA changes do not necessarily translate into changes of protein levels. The authors decided to validate RNA levels through RT-PCRs instead of a single western blot of HXK1 in one of the conditions where the corresponding gene levels are changed? The question remains.

The authors claim in the abstract that: 'This research … presents potential strategies for enhancing healthspan and delaying the onset of age-related diseases. These findings have the potential to revolutionize our approach to implementing these interventions'. This reviewer finds this central statement very strong without any validation of RNASeq data.

Thank you for your suggestions. We have amended the abstract accordingly.

5. The point relates to point 4. I understand and agree with the authors that comprehensive analyses are not the scope of the paper. That's why I suggested only validating a few candidates.

Thank you for understanding and acknowledging our provided validation results.

6. The derived graph plot provided by the authors is acceptable. The authors have addressed the point.

Thank you for confirming the acceptability of the graph plot.

7. The authors have addressed the point.

Thank you for confirming that the point has been addressed.

8. I appreciate the effort of authors to improve the figures but the point remains. Fonts can be very small and difficult to read throughout. For example, see labels/fonts of categories in Figure 2e.

Thank you for the suggestions. We have included high-quality figures in the revised submission.

9. Many thanks for the data deposition, I am sure this will be checked by the journal too.

Thank you for your kind review and suggestions for improving our manuscript.

Reviewers' comments:

Reviewer #3 (Remarks to the Author):

The authors have done text changes that have been recommended.

However, they have ignored further suggestions and comments using the phrase 'beyond the scope of the manuscript' although a justification for the given points has been provided by this reviewer.

Specifically:

Point 3: not addressed as 'beyond the scope'. Justification for this ask has been provided. The ask was for validation of 1-2 results only to solidify the claims from the RNA-seq results, extensive discussion and development of scenarios.

We apologize for the miscommunication. We have now performed Western blot analysis for Glk1 protein (Figure S3f), showing a consistent decrease following rapamycin treatment, supporting our RNA-seq findings. This targeted validation addresses your concern. We have added this data and western blot method to the revised manuscript.

Point 4: while the authors made a text change in the abstract to remove very strong statements, they have ignored the first part of the point. They have decided to provide RT-PCR data in the previous round of revision, instead of a couple simple western blots for the proteins of interest. This is very surprising. I have raised the point once again and it is still not addressed.

We regret that our previous response on protein validation was unsatisfactory. As mentioned in Point 3, we have performed Western blot analysis for Glk1 protein (Figure S3f), directly addressing your repeated request for protein validation of a key gene. This links our mRNA and protein expression data.

Point 5: not addressed. I agree with the authors that a comprehensive protein-level analysis is not required for the manuscript. I have asked only for a couple of validations related e.g. to HXK1. I've never agreed with the authors on point 5.

To address this, we have now performed Western blot analysis for Glk1 protein (Figure S3f), which, like Hxk1, is a key glycolytic enzyme. Due to unforeseen circumstances, including our lab's relocation to a different institution and the subsequent time required to re-establish our yeast laboratory, we faced significant delays in generating tagged strains for Western blot analysis. We received the c-9xMYC plasmid and have been working to generate c-MYC tagged strains for both Hxk1 and Glk1. We have, to date, successfully generated the Glk1 tagged strain. Given the reviewer's request for 1-2 protein validations, we believe that providing the Glk1 Western blot data, which shows a similar pattern in our RT-PCR data to Hxk1, adequately addresses the core concern in this revision.

Point 8: while high-quality images are provided, the fonts and arrangements of figures still need attention. They are very populated and busy with tiny fonts.

We appreciate your feedback on the figures. We acknowledge the font sizes and arrangements need improvement. While significant font size increases are technically challenging, we can split complex figures if required for publication to improve readability.

Remarks to the Author:

I have read through the revised manuscript and rebuttal letter from the authors. And I am concerned with some important issue before approving the publication of this paper.

As I emphasized in the previous review report, while the title of this manuscript is “Systematic transcriptomics analysis of calorie restriction and rapamycin unveils their synergistic interaction in prolonging cellular lifespan”, the experimental design and data analysis deviate from this theme. Specifically, the experimental design only collects very young cells (5 hr for mitotic and 72 hr for post-mitotic, respectively) for RNA-seq, which I belive is a major flaw of this paper. Only transcriptome profiles of these very young cells under CR and/or RM are neither adequate nor sufficient to unveil their synergistic interaction in prolonging cellular lifespan (Aging is a complicated biological proces, and not just one time point).

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This was also mentioned by the Reviewer #3: “The experimental design is very poor... the authors should provide growth curves and use appropriate timepoints for the different conditions examined that are comparable (e.g. 1, 2, 3, 4 days etc following entrance to stationary phase). I find this to be a major problem of the manuscript as all subsequent data are based on this flawed scheme.”

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