

Small RNA profiling reveals inflammatory and mitochondrial changes in aging β cells and islet macrophages

Corresponding Author: Dr Cristina Cosentino

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)

Guay et. al nicely show that aging induces a comprehensive remodeling of the small non-coding RNA (sncRNA) landscape in pancreatic β -cells, affecting miRNAs, isomiRs, tRFs, and piRNAs. This remodeling shifts β -cells toward a pro-inflammatory, less proliferative, and more mature phenotype, with specific changes in miRNA and isomiR expression. Notably, mitochondrial tRFs (mt-tRFs) are significantly upregulated in aged β -cells, potentially impacting mitochondrial function. While senescent β -cells share some sncRNA changes with aged cells, they also exhibit unique signatures, such as the specific upregulation of miR-184-3p. Pro-inflammatory cytokines can recapitulate key sncRNA changes seen in aging and senescence, indicating inflammation as a driver of these alterations. Furthermore, aging induces a shift in islet-resident macrophages (iMACs) toward an anti-inflammatory state, suggesting a protective adaptation. Importantly, the sncRNA changes observed during aging overlap significantly with those seen in models of both Type 1 and Type 2 diabetes, positioning aging as a key risk factor for diabetes development.

This study presents a novel and comprehensive analysis of small non-coding RNA (sncRNA) changes in aging pancreatic β -cells and islet-resident macrophages (iMACs). It reveals that aging induces a profound, multi-layered reprogramming of the sncRNome, including the specific upregulation of mitochondrial tRFs and distinct miRNA/isomiR signatures linked to inflammation and reduced proliferation.

This is a well-executed, technically sophisticated study that provides a comprehensive and high-resolution profiling of small non-coding RNAs (sncRNAs) in pancreatic β -cells and islet-resident macrophages (iMACs) across three age groups in male C57BL/6J mice. The work leverages FACS-sorted cells, small RNA sequencing, qPCR validation, and in vitro cytokine stimulation models to dissect age- and senescence-associated changes in miRNAs, isomiRs, tRFs, and piRNAs.

The manuscript is well-structured, data-rich, and highly relevant to the fields of aging, diabetes, and islet biology. It offers a valuable resource for future studies on sncRNA regulation in metabolic health and disease. However, several critical points require attention before publication.

Major Recommendations

1. For clarity, it would be helpful to specify the number of biological replicates (i.e., animals) used for each sequencing experiment and whether technical replicates were included. Additionally, given the small sample size ($n=3$ per group), using a more stringent FDR threshold (e.g., ≤ 0.05) for the primary differential expression analysis might enhance confidence in the results. If applicable, it would also be useful to mention whether batch effects were assessed or corrected for during the analysis.
2. The observation that primarily mitochondrial tRNAs and tRFs were detected is intriguing. This could reflect biological changes, but it may also be influenced by technical factors such as differences in post-transcriptional modifications or library preparation efficiency between cytosolic and mitochondrial tRNAs. A brief discussion of this potential technical bias and its implications for interpreting the data would be helpful for readers.
3. The use of cytokine exposure to model aging is a clever approach. To strengthen the interpretation, it might be worth discussing in more detail the limitations of the short-term treatment and washout period in fully recapitulating the chronic, low-grade inflammation of aging. Clarifying why dispersed islets were used instead of intact islets (and the rationale for this

choice) as well as more details on culture conditions would improve reproducibility. It would also be reassuring to know whether cell viability was assessed after the 6-day culture period, as prolonged culture (of single cells) could influence sncRNA profiles. Exploring longer or repeated exposure paradigms in future work could further validate the model.

4. The manuscript draws strong functional links between specific miRNAs and processes like proliferation or maturation based on literature meta-analysis. While this is a valuable approach, it's important to acknowledge that miRNA functions can be context-dependent and sometimes contradictory across different models. Using more cautious language (e.g., "associated with" or "reported to regulate") and highlighting potential context-specific roles would make the interpretation even more robust.

Minor Recommendations

1. While the functional annotations provide valuable context, the results section currently reads as a list of associations. To enhance readability and engagement, it would be beneficial to weave these functional insights into a more narrative flow perhaps by highlighting key biological themes (e.g., the shift toward inflammation, the decline in regenerative capacity, or the mitochondrial remodeling) that emerge across the data. This would help readers grasp the bigger picture and make the findings more compelling and memorable.

2. Including a brief discussion on the piRNA data (even if not deeply explored) and the potential translational relevance of these findings to human aging and diabetes would add depth to the discussion.

3. It would be interesting to know whether the overlap between cytokine-induced and aging-related changes extends beyond isomiRs to canonical miRNAs as well. A brief comparison would provide a more complete picture.

4. A short note on how the specific miRNAs and tRFs selected for qPCR validation in other tissues were chosen (e.g., based on magnitude of change, functional relevance, or novelty) would improve transparency.

5. Given that the current data are from mouse models, a brief discussion on whether similar sncRNA changes have been observed in human islets or aging studies would be very valuable. Are there conserved patterns between mouse and human aging? This would help contextualize the findings for broader relevance.

6. The discussion of the miR-132/212 cluster in the context of insulin resistance in metabolic tissues is well-placed. It might be worth briefly noting that while β -cells are insulin-responsive, the mechanisms of insulin resistance in β -cells may differ from those in liver or muscle, and this could influence the interpretation of miRNA function in this context.

Reviewer #2

(Remarks to the Author)

The authors characterized small non coding RNA expression in mouse derived β -cells and islet-resident macrophages (iMACs) in different ages. They found distinct signatures in the senescent population of these cells, distinguished via SA-beta-gal staining.

In abstract, in line 52 the authors should be more specific and name a number of changing tRFs and miRNAs or related pathways.

In line 133 and 147 specific antibodies used for FACS strategies for sorting of β -cells and iMACS should be mentioned.

For RNAseq analysis, was there a threshold employed for minimal library size? What is the percentage of mapped reads of miRNAs and tRFs?

For Fig.1 f-m, a color legend for the heatmap plots is missing.

In line 269, the authors should be more specific which isomiRs of miR-211-3p etc are deregulated. Is there a pattern in the isomiR classes that are deregulated, e.g. 3' or 5' alterations or base switching?

Line 247: What is the overlap between age-regulated miRNAs and isomiRs and the cytokine regulated ones? This could be illustrated in a Venn diagram.

Line 301: Add a reference for modulations tRNA nucleotides affecting library preparation.

Additionally, a analysis looking into the ratio between full length tRNA transcripts and their tRFs derived from these transcripts would be very interesting here to see whether the expression levels of tRFs corresponds with the specific full length tRNA for all its fragments or just specific fragments remain.

"Interestingly, several mitochondrially-encoded tRFs were upregulated in 22-month-old β -cells" – give precise numbers of tRFs

In line 314 should be an explanation why these specific miRNAs and tRFs were picked for validation experiments. Was there a specific expression threshold or FC cut off employed?

Why were 7/8 months old animals chosen for the analysis of senescent cells? There is no reference for this age in the initial data. For this mouse cohort the sex of the mice is missing. There should also be an indication how many cells were sorted for these two populations. And a comparison for the sorted Beta-cells at this age without the sorting into subgroups to enable and understanding of the contribution of the different subpopulations to the entire population.

In line 349 and following the wording induced is misleading and should be replaced by e.g. upregulated.

What is the overlap between deregulated tRFs in the senescent cells and aging?

For the characterization of the cytokine treated islet cells a staining with Beta-Gal, which was previously used to identify senescent cells is missing. There should be a validation with either this or with EdU staining to confirm senescence induction in these cells.

Line 537: There is no through analysis of the piRNA data set in the current version of the paper. This should be included with figures and statistics in the results if the authors claim to a detailed analysis of piRNAs.

Overall, a nice and interesting study however since only male mice were used in the entire study not representative for population top hits and claims need to be validated in a female population.

Reviewer #3

(Remarks to the Author)

This manuscript presents a comprehensive profiling of small non-coding RNAs (sncRNAs) in pancreatic β -cells and islet macrophages during aging. The authors perform small RNA sequencing on FACS-sorted β -cells and islet macrophages (iMACs) from 3-, 12-, and 22-month-old male C57BL/6J mice and further analyze senescent (SA- β -gal⁺) β -cells as well as cytokine-treated islets. They report age-associated remodeling of several sncRNA classes, including miRNAs, isomiRs, piRNAs and tRNA-derived fragments (tRFs), and propose that aging is associated with an inflammatory miRNA signature in β -cells, an induction of mitochondrial tRFs, and a shift toward an anti-inflammatory or tissue-repair phenotype in aged islet macrophages.

The dataset is potentially valuable and the topic is timely, as the role of regulatory RNAs in metabolic aging and β -cell dysfunction has received increasing attention in recent years. Profiling sncRNA dynamics in defined islet cell populations across aging could provide an important resource for the field. Nevertheless, several aspects of the study require clarification or additional experimental support before the conclusions can be considered robust. In its current form the work remains largely descriptive, and several interpretations extend beyond the evidence presented.

Overall, this study provides an interesting dataset describing sncRNA remodeling in aging islet cells and could represent a useful resource for the field. However, clearer reporting of the experimental design, additional technical validation of key RNA species, and stronger functional evidence linking these RNAs to β -cell physiology would substantially strengthen the manuscript. With revisions as indicated, and a more cautious interpretation of the results, the work could make a valuable contribution to our understanding of regulatory RNA networks in islet biology and metabolic aging.

Major.

1. Experimental design and statistical reporting. The authors should clearly report the exact number of independent RNA-seq libraries and biological replicates used for each dataset, including the samples used for qPCR validation. Please clarify these numbers both in the Methods section and directly in the figure legends so that the robustness of the analyses can be assessed. The authors should also justify the differential expression criteria used in the study (fold change >1.5 and FDR <0.1), which appear relatively permissive given the large number of transcripts tested across multiple sncRNA classes. In addition, report the total number of tested transcripts per RNA category (miRNAs, tRFs, piRNAs, etc.) and the number retained after statistical filtering. In several cases the qPCR validation experiments show only trends rather than statistically significant changes, for example for miR-34a-5p, mitochondrial 12S/16S fragments, and several tRFs (Fig. 3 and Supplementary Fig. 3; $p \approx 0.06-0.1$). Similarly, temper the associated conclusions and clearly acknowledge when validation experiments do not reach statistical significance.

2. From descriptive to mechanism in β -cells. A broader limitation of the manuscript is the lack of functional evidence linking the identified RNAs to β -cell aging or dysfunction. Because the manuscript frequently interprets these RNA changes in terms of β -cell function, inflammation, or metabolic decline, the authors need to provide additional phenotypic characterization of the same cohorts of mice used for sequencing. Specifically, they should measure and report functional parameters such as glucose-stimulated insulin secretion, basal insulin secretion, insulin content, proliferation markers (e.g. Ki67), and classical senescence markers including p16, p21 or SA- β -gal activity. These phenotypes should be correlated where possible with the expression of key RNAs identified in the study. Previous studies have shown that aging β -cells display increased expression of senescence regulators such as p16Ink4a and p21, reduced proliferative capacity, and altered mitochondrial metabolism (Aguayo-Mazzucato et al., Cell Metabolism, 2017; Helman et al., Nature Communications, 2016).

3. The manuscript highlights several miRNAs and tRFs as potential regulators of β -cell "inflammaging," including miR-146a-5p, miR-142-3p/5p, miR-181a/b, miR-143/145 and tRFs such as 5'tRF-Gly(GCC) or mitochondrial fragments derived from tRNA-Leu and tRNA-Ser. To support these claims, perform gain- or loss-of-function experiments for at least a subset of these candidates in primary islets or β -cell models. The authors need to assess relevant functional outcomes such as insulin secretion, apoptosis, or levels of senescence markers following perturbation of these RNAs. Previous work has demonstrated that individual miRNAs can profoundly influence β -cell biology. For example, miR-375 regulates insulin secretion and β -cell mass (Poy et al., Nature, 2004), and disruption of miRNA processing in β -cells leads to severe defects in β -cell function and survival (Latreille et al., Genes & Development, 2014), plus many studies from the authors' own laboratory. I recommend using similar approaches to determine whether any of the candidate RNAs, as identified in this study, directly contribute to aging phenotypes.

4. tRF detection and mitochondrial interpretation. The analysis of tRNA-derived fragments requires further clarification. Please provide additional information on the detection and classification of tRFs, including the length distribution of detected fragments, the relative representation of mitochondrial versus nuclear tRFs, and the number of fragments passing statistical thresholds. Because the sequencing strategy did not include removal of tRNA modifications and the mapping strategy appears to require perfect sequence matches, please discuss the potential biases introduced by these methodological choices. Independently validate several representative fragments (for example 5'tRF-Gly(GCC), mt-5'tRF-Ser(GCT), or mt-itRF-Leu(TAA)) using orthogonal approaches such as Northern blotting or ligation-based qPCR. Until such validation is provided, moderate the claim that mitochondrial tRFs are globally induced during aging.

5. Inflammation vs aging causality. The cytokine treatment experiments are interpreted as a model of aging-related inflammation, yet the relationship between these conditions is not fully demonstrated. Acute cytokine exposure may reflect stress responses rather than the chronic inflammatory environment associated with aging. Please quantify the overlap between cytokine-regulated sncRNAs and those altered in aged or senescent β -cells. Present this analysis explicitly, for example using overlap statistics or enrichment analyses. If the cytokine treatment is intended to mimic aspects of aging or

senescence, then please provide functional measurements such as insulin secretion or senescence marker expression under these conditions.

6. iMAC phenotype. The proposed phenotype of aged islet macrophages also requires additional support. The authors suggest that aged iMACs may adopt an anti-inflammatory or tissue-repair phenotype based largely on miRNA expression patterns. Complementary expression analysis of classical macrophage activation markers (including Tnf, Il1b, Il6 and Nos2 for inflammatory states and Il10, Arg1 or Mrc1 for repair-associated states) would provide a more direct assessment of macrophage polarization. If feasible, simple ex vivo assays measuring phagocytic or efferocytic activity could further strengthen the proposed interpretation.

7. Figure-specific aspects. In the differential expression analyses (Fig. 1–2), the authors should clearly indicate the statistical thresholds used, the number of tested transcripts per RNA class, and the number of significant hits after correction for multiple testing. In the validation experiments (Fig. 3 and Supplementary Fig. 3), some of the reported changes appear to approach but not reach statistical significance, and the corresponding conclusions should be adjusted accordingly. In the comparison between β -cells and iMACs (Fig. 4), it would also be useful to clarify how normalization was performed when comparing two cell types with very different baseline RNA expression profiles. Finally, the cytokine treatment experiment (Fig. 5) would benefit from a quantitative comparison with the aging datasets to better establish the relationship between inflammatory stimulation and age-associated RNA remodeling.

8. Scope and Limitation. Finally, several limitations of the study should be more explicitly acknowledged in the Discussion. The work is restricted to male C57BL/6J mice, metabolic phenotyping of the animals is limited (body weight and fed glycemia only), and most conclusions are based on correlative analyses. In addition, the Discussion occasionally overstates the potential translational implications of the findings, particularly the suggestion that these RNA signatures might help predict individuals who will age metabolically healthy versus those who will develop metabolic disease. Given the current data, such claims appear premature.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for their thorough and thoughtful responses to my comments. I appreciate the extensive revisions made to the manuscript, and I believe that my major concerns have been satisfactorily addressed. The additional analyses, clarifications, and discussion have substantially strengthened the manuscript.

I only have a few minor remarks that should be addressed before publication:

Figures:

The figure legend for panels 2c,d appears to be missing.

Figure 4: The symbols in panels 4a and 4e are not displayed correctly.

Figure labeling: The labels in several panels (e.g., Figures 2d and 4b) are very small and difficult to read. Increasing the font size would improve readability.

Figure 5b: It would be helpful to provide representative images.

Supplementary Figure 1a,b: The labeling appears to be slightly misaligned and could be adjusted for improved clarity.

Overall, I congratulate the authors on this comprehensive study and thank them for carefully addressing the reviewers' comments.

Reviewer #2

(Remarks to the Author)

All comments were addressed. Good work.

Reviewer #3

(Remarks to the Author)

The authors have addressed many of the concerns raised. The manuscript is certainly improved, and the addition of functional experiments, particularly regarding mt-tRFs, provides valuable insights. However, some critical points remain that require clarification, or more cautious interpretation, before the work can be considered robust.

Major points

1. Please clearly distinguish the acute 24-hour cytokine small-RNA sequencing dataset from the separate cytokine-plus-

washout model used for the functional experiments in Figure 5. The number of independent biological replicates for the 24-hour sequencing dataset should be stated explicitly in the Methods and Figure 2 legend, since the RRHO analysis depends on it. Please also clarify in the Manuscript that the RRHO analysis evaluates overlap between acute cytokine and aging-associated sncRNA changes, whereas the washout experiment provides targeted phenotypic validation and does not recapitulate the complete aging sncRNA profile.

2. The new mt-tRF loss-of-function experiments are strong and really useful, but they were performed in young islet cells and MIN6B1 cells, not in the aged β -cells where the RNA changes were identified. Please either provide direct phenotyping of the aged cohorts or clarify throughout the manuscript that the age-associated RNA changes remain correlative. Revise the interpretation from “mt-tRFs maintain mitochondrial function during aging” to “mt-tRF perturbation can influence mitochondrial function and insulin secretion in the tested cellular models.”

3. The manuscript states that no significant age-dependent changes were detected at 12 months, but then notes that all detected full-length tRNAs were “upregulated” at 22 months. Please clarify whether “upregulated” means statistically significant or simply higher relative sequencing counts. Since this method cannot reliably measure absolute RNA levels or the exact relationship between full-length tRNAs and their fragments, please use more cautious wording such as “higher relative sequencing abundance” or “age-associated enrichment.”

4. The manuscript continues to infer an “anti-inflammatory” or “tissue-repair” phenotype for aged iMACs based primarily on miRNA patterns, without direct measurements of macrophage activation markers or functional assays like phagocytosis. While the added limitation statement is helpful, the Abstract and Results remain strong in their inferences. Please either add direct marker/functional data or revise the language to state: “The aged iMAC miRNA profile was consistent with a possible anti-inflammatory or homeostatic remodeling, but this phenotype was not directly established.”

Minor Text Corrections:

- Correct “FAC-sorted” to “FACS-sorted” throughout;
- Correct “reduced of Sirt1” to “reduced Sirt1” or “reduced expression of Sirt1”;
- Ensure the term “aging-or senescence-like” includes proper spacing or hyphenation;
- Harmonize nomenclature for “miR-181a-5p” and “miR-181b-5p”;
- Minor figure-legend labeling problem in Figure 2;
- Please consistently use absolute fold-change notation to avoid ambiguity;
- Please clarify the significance threshold in figure legends to ensure p-values between 0.05 and 0.1 are visually and textually distinguished from statistically significant values ($p < 0.05$).

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Reviewer #1 (Remarks to the Author):

Guay et. al nicely show that aging induces a comprehensive remodeling of the small non-coding RNA (sncRNA) landscape in pancreatic β -cells, affecting miRNAs, isomiRs, tRFs, and piRNAs. This remodeling shifts β -cells toward a pro-inflammatory, less proliferative, and more mature phenotype, with specific changes in miRNA and isomiR expression. Notably, mitochondrial tRFs (mt-tRFs) are significantly upregulated in aged β -cells, potentially impacting mitochondrial function. While senescent β -cells share some sncRNA changes with aged cells, they also exhibit unique signatures, such as the specific upregulation of miR-184-3p. Pro-inflammatory cytokines can recapitulate key sncRNA changes seen in aging and senescence, indicating inflammation as a driver of these alterations. Furthermore, aging induces a shift in islet-resident macrophages (iMACs) toward an anti-inflammatory state, suggesting a protective adaptation. Importantly, the sncRNA changes observed during aging overlap significantly with those seen in models of both Type 1 and Type 2 diabetes, positioning aging as a key risk factor for diabetes development.

This study presents a novel and comprehensive analysis of small non-coding RNA (sncRNA) changes in aging pancreatic β -cells and islet-resident macrophages (iMACs). It reveals that aging induces a profound, multi-layered reprogramming of the sncRNome, including the specific upregulation of mitochondrial tRFs and distinct miRNA/isomiR signatures linked to inflammation and reduced proliferation.

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The manuscript is well-structured, data-rich, and highly relevant to the fields of aging, diabetes, and islet biology. It offers a valuable resource for future studies on sncRNA regulation in metabolic health and disease. However, several critical points require attention before publication.

We thank the reviewer for the overall positive comments on our study and for identifying critical points that we addressed in our revision and in our response to the reviewer's comments.

Major Recommendations

1. For clarity, it would be helpful to specify the number of biological replicates (i.e., animals) used for each sequencing experiment and whether technical replicates were included.

Additionally, given the small sample size ($n=3$ per group), using a more stringent FDR threshold (e.g., ≤ 0.05) for the primary differential expression analysis might enhance confidence in the results.

If applicable, it would also be useful to mention whether batch effects were assessed or corrected for during the analysis.

We thank the reviewer for these valuable suggestions. We have now included detailed information on the number of biological replicates in [lines 302 and 348](#), as well as in all figure legends. Biological replicates correspond to β -cells and islet macrophages isolated by FACS from

independent pancreatic islet pools. For 3-month-old mice, islets from four mice were pooled per replicate, whereas for 12- and 22-month-old mice, islets from 2-3 animals were pooled per replicate. To provide greater clarity regarding the experimental design, we have added a supplementary table (**Suppl. Table 1**) summarizing the islet pooling strategy. All replicates used in the RNA-seq analyses were biological replicates; no technical replicates were performed.

As indicated in Supplementary table 1, four biological replicates per age group were used for small RNA-seq profiling. Following the reviewer's suggestion, we reanalyzed the data using more stringent thresholds ($FDR \leq 0.05$ and Fold change ≥ 2) to define significantly modulated sncRNAs. These results were applied to update the data in **Suppl Figure 1.h**. In addition, we included a new Venn diagram illustrating the overlap of differentially expressed miRNAs between 12- and 22-month-old β -cells using these more stringent cut-offs **Suppl Figure 1.i**. The updated lists were also used for functional enrichment analysis. The enriched pathways remained largely unchanged, indicating that the main biological conclusions are not affected by the choice of statistical thresholds. We have now clarified in [lines 325-328](#) that less stringent cut-offs were applied in certain enrichment and metanalysis approaches to capture modest but potentially biologically relevant changes in sncRNA expression.

Regarding potential batch effects, all islet cell samples were collected and processed in parallel on the same day, with biological replicates representing independent pools of islets obtained from different animals. To further address this point, we have added principal component analysis of the β -cell small RNA-seq dataset, which shows clustering of samples according to animals age (**Suppl. Figure 1.e**, [line 303](#)).

2. The observation that primarily mitochondrial tRNAs and tRFs were detected is intriguing. This could reflect biological changes, but it may also be influenced by technical factors such as differences in post-transcriptional modifications or library preparation efficiency between cytosolic and mitochondrial tRNAs. A brief discussion of this potential technical bias and its implications for interpreting the data would be helpful for readers.

We thank the reviewer for this insightful comment. We acknowledge that structural differences between mitochondrial and cytosolic tRNAs, as well as their corresponding tRFs, may influence their detection and quantification in small RNA sequencing analyses. To address this issue, we performed additional small RNA-seq analysis using samples from β -cells of 3- and 12-month-old female mice after enzymatic removal of secondary nucleotide modifications that might interfere with reverse transcription. Notably, also in this dataset, mitochondrial tRFs were induced with aging (Suppl. Figure 2 of the revised manuscript). The findings are described in [lines 428-437](#), and [443-452](#). The new findings support the robustness of our observations and indicate that the age-associated induction of mitochondrial tRFs is not primarily driven by biases introduced during library preparation. We have now expanded the discussion of this point in the revised manuscript [lines 444-446, 715-719, 733-737](#).

3. The use of cytokine exposure to model aging is a clever approach. To strengthen the interpretation, it might be worth discussing in more detail the limitations of the short-term treatment and washout period in fully recapitulating the chronic, low-grade inflammation of aging.

It would also be reassuring to know whether cell viability was assessed after the 6-day culture period, as prolonged culture (of single cells) could influence sncRNA profiles.

Exploring longer or repeated exposure paradigms in future work could further validate the model.

We are grateful to the reviewer for suggesting that we clarify the rationale and limitations of our in vitro model. We have now expanded the description of the model in the Results section [lines 548-561](#) and extended the Discussion ([lines 752-761](#)) to emphasize that the washout period used in our study captures only the short-term persistence of inflammatory effects. As such, it cannot fully recapitulate the cumulative and progressive nature of aging-associated inflammation, which develops over months or years. Therefore, our findings should be interpreted as reflecting the ability of inflammatory stress to induce aging-like or persistent stress-associated features in β -cells, rather than as a comprehensive model of physiological aging.

The use of dispersed mouse islets rather than intact islets was a deliberate experimental choice. Previous observations from our lab indicated that prolonged culture of whole islets results in a loss of structural integrity and the development of central necrosis. Dispersing the islets ensured more homogenous access of cells to nutrients and oxygen under standard cell culture conditions, thereby improving viability and reducing culture-related effects. To assess viability, we performed Hoechst staining and have now included these data in **Figure 5b** and [lines 554-556](#). Acute pro-inflammatory cytokine treatment led to a 2-fold increase in the number of pyknotic nuclei, indicative of cell death, that was closed to significance ($p=0.053$). Interestingly, following the wash-out period, the number of cells showing pyknotic nuclei was no longer significantly different from control conditions, indicating that cell viability was recovering from cytokine treatment.

4. The manuscript draws strong functional links between specific miRNAs and processes like proliferation or maturation based on literature meta-analysis. While this is a valuable approach, it's important to acknowledge that miRNA functions can be context-dependent and sometimes contradictory across different models. Using more cautious language (e.g., "associated with" or "reported to regulate") and highlighting potential context-specific roles would make the interpretation even more robust.

We thank the reviewer for raising this point. We acknowledge that miRNA function can be strongly influenced by the cellular and environmental context. As suggested by the reviewer, we have now adopted a more cautious language when describing miRNA functional involvement.

Minor Recommendations

1. While the functional annotations provide valuable context, the results section currently reads as a list of associations. To enhance readability and engagement, it would be beneficial to weave these functional insights into a more narrative flow perhaps by highlighting key biological themes (e.g., the shift toward inflammation, the decline in regenerative capacity, or the mitochondrial remodeling) that emerge across the data. This would help readers grasp the bigger picture and make the findings more compelling and memorable.

Following the revision process, we have now included potential interpretations of the results together with functional experiments that might improve the narrative flow of the manuscript.

2. Including a brief discussion on the piRNA data (even if not deeply explored) and the potential translational relevance of these findings to human aging and diabetes would add depth to the discussion.

As suggested by the reviewer, we have included in the revised manuscript a brief discussion of the piRNA data and of their potential relevance in [lines 401-407](#).

3. It would be interesting to know whether the overlap between cytokine-induced and aging-related changes extends beyond isomiRs to canonical miRNAs as well. A brief comparison would provide a more complete picture.

We thank the reviewer for suggesting a more accurate comparison between the aging-induced changes and those triggered by acute inflammation. To address this point, we employed the R-package RedRibbon to perform a Rank Rank Hypergeometric Overlap (RRHO) analysis, a method designed to reveal overlapping features between two differential expression datasets. This analysis highlighted notable inverse correlations in changes of both miRNAs and isomiRs, as reflected in the up-down and down-up quadrants of the RRHO plot (Figure 2.c). However, a significant overlap was observed among commonly upregulated miR/isomiRs, which included the well-characterized inflamma-miRs miR-146-5p and miR-143-3p. Applying a threshold of $FDR < 0.05$, we further identified miR/isomiR changes shared across both datasets, encompassing species upregulated in aging and downregulated in cytokines, as well as those downregulated in aging and upregulated in cytokines (Figure 2.d). The results are described in the Results section [lines 388-400](#).

4. A short note on how the specific miRNAs and tRFs selected for qPCR validation in other tissues were chosen (e.g., based on magnitude of change, functional relevance, or novelty) would improve transparency.

We thank the reviewer for this comment. We now clarified in the manuscript (see lines 355-357) that the miRNAs selected for validation were chosen based on their demonstrated roles in relevant pathways modulated in aging: inflammation (miR-146-5p); aging and cell proliferation (miR-181b-5p) and insulin sensitivity and glucose homeostasis (miR-143-3p and miR-145-5p). In addition, miR-34a-5p was included given its well-established association with aging across multiple tissues. Regarding tRF selection, we chose to validate mitochondrial and cytosolic tRFs previously reported to be downregulated in obesity and T2D and upregulated in a T1D model, namely (mt-tRF^{SER(GTC)}, mt-tRF^{LEU(TAA)} and the stress-associated 5'tRF^{Gly(GCC)}) ([lines 472-477](#))

5. Given that the current data are from mouse models, a brief discussion on whether similar sncRNA changes have been observed in human islets or aging studies would be very valuable. Are there conserved patterns between mouse and human aging? This would help contextualize the findings for broader relevance.

We thank the reviewer for this suggestion. To our knowledge, sncRNA profiling in β -cells or islets of elderly individuals has not been performed yet and will be essential to identify which sncRNAs are commonly changed during murine and human β -cell aging (discussed in limitations: [lines 777-779](#)). However, miR-146a has been previously linked to human inflammation and aging ([text line 324](#)) and some aged-associated miRNAs have been previously identified as deregulated in diabetic conditions in both rodent models and patients, exhibiting conserved functions. All relevant studies are listed in the literature metanalysis in **Suppl. Data 6**. Moreover, a recent study observed an increased disparity between mRNA and protein levels in different organs in elderly humans, suggesting that post-transcriptional regulators like sncRNA could play an important role in the acquisition of the aging phenotype (Ding, Y. et al, *Cell* 2025, [lines 779-780](#)). Regarding other classes of sncRNAs, we have recently reported the modulation of tRFs in islets from T2D patients (Cosentino et al. 2026, [lines 420-423](#)) and the role of mt-tRFs to regulate insulin secretion in

human islets (Jacovetti et al 2024, [lines 592-594](#)) . However, the tRF association with human islet aging remains to be investigated.

6. The discussion of the miR-132/212 cluster in the context of insulin resistance in metabolic tissues is well-placed. It might be worth briefly noting that while β -cells are insulin-responsive, the mechanisms of insulin resistance in β -cells may differ from those in liver or muscle, and this could influence the interpretation of miRNA function in this context.

[In the revised manuscript, we briefly describe the differences in insulin responses between \$\beta\$ -cells and metabolic tissues.](#)

Reviewer #2 (Remarks to the Author):

The authors characterized small non coding RNA expression in mouse derived β -cells and islet-resident macrophages (iMACs) in different ages. They found distinct signatures in the senescent population of these cells, distinguished via SA-beta-gal staining.

1. In abstract, in line 52 the authors should be more specific and name a number of changing tRFs and miRNAs or related pathways.

[We thank the reviewer for the suggestion; the abstract has been changed accordingly.](#)

2. In line 133 and 147 specific antibodies used for FACS strategies for sorting of β -cells and iMACS should be mentioned.

[We have now added the catalog number information of the antibodies used for iMAC sorting in the method section \(\[lines 143-156\]\(#\)\). In our FACS-sorting strategy \(**Suppl. Figure 1.d**\), \$\beta\$ -cells are sorted based on their autofluorescence. As mentioned in the Method section, iMAC population is first identified using specific antibodies, then green autofluorescence is used to obtain a fraction enriched in \$\beta\$ -cells.](#)

3. For RNAseq analysis, was there a threshold employed for minimal library size?

[For RNAseq analysis we did not apply any threshold for library size being all of them in between 190 and 230 bp average size as measured with Agilent fragment analyzer. On the other hand, the minimum sequencing depth was set at 25 million reads/library. This detail has been added to the method section \(\[lines 184-187\]\(#\)\).](#)

4. What is the percentage of mapped reads of miRNAs and tRFs?

[As requested by the reviewer, we added the following details:](#)

[- Expression filtering methods adopted during small RNA-seq analysis. Lines --](#)

[- Unique counts before expression filtering and raw counts retained after filtering and mapped to miRNAs, tRFs or other known chromosomal locations \(Suppl. Figure 1.f-g ; Suppl. Figure 2.a-b; Suppl. Figure 8.a-d; and text \[lines: 309, 350, 404, 430\]\(#\)\)](#)

[An explanation for the differences in the miRNA – tRF coverage between the male \$\beta\$ -cell dataset and the female \$\beta\$ -cell dataset, which was obtained after depletion of RNA modifications, is provided in \[lines 349-352\]\(#\) . In summary: removal of RNA modifications drastically reduced the % of reads mapping to miRNAs \(from \$\approx 70\%\$ in the male dataset to \$\approx 2\%\$ in the female dataset\) while increasing the tRNA-mapping counts from 1-5% to 7-14%. Despite these differences, the miRNA](#)

coverage in the female dataset was sufficient to perform differential expression analysis, presenting similar number of hits of the male dataset.

5. For Fig.1 f-m, a color legend for the heatmap plots is missing.

We thank the reviewer for spotting this omission. We have now added a color bar.

6. In line 269, the authors should be more specific which isomiRs of miR-211-3p etc are deregulated. Is there a pattern in the isomiR classes that are deregulated, e.g. 3' or 5' alterations or base switching?

We have now added the information about the specific β -cell isomiR signature that is deregulated in our dataset ([lines 379-380](#)). Among the deregulated isomiRs, we noticed a prevalence of 3' alterations (trimming or additions) and base switching, while 5' alterations were less represented in isomiRs deregulated with aging. In addition, we have added an explanation on the isomiR nomenclature methods used by the RNA-seq analysis portal (Qiagen) ([lines 206-208](#)).

7. Line 247: What is the overlap between age-regulated miRNAs and isomiRs and the cytokine regulated ones? This could be illustrated in a Venn diagram.

We thank the reviewer for pointing out the need for more extensive comparisons of the two datasets. As noted, the inflammatory profile established during aging likely reflects low-grade chronic inflammation and probably differs from the acute stress response induced by direct exposure to elevated cytokine concentrations. To explore this further, we performed a Rank-Rank Hypergeometric Overlap (RRHO) analysis comparing differentially expressed miRNAs and isomiRs in aging (22 months vs young) and in response to cytokine treatment (cyto vs ctr). Although significant correlations were found among entries modulated in the same direction under both conditions, this analysis clearly highlighted differences between the iso/miRNome signatures defined by the 2 conditions. Applying threshold of FDR <0.1, we identified miRNAs and isomiRs detected and modulated in both datasets. Among these, 8 small RNAs were modulated in the same direction, including miR-146-5p and miR-143-3p. Fifteen miR/isomiR molecules were downregulated by cytokines but upregulated in aging, including miR-409-3p, previously reported to be downregulated in plasma from T1D patients and upregulated in T2D patients. Finally, 10 miR/isomiRs were upregulated by cytokines but downregulated during aging, including miR-21a-5p, previously associated with cytokine-induced apoptosis. These results are shown in Figure 2.c-d and described in [lines 388-400](#).

8. Line 301: Add a reference for modulations tRNA nucleotides affecting library preparation.

We thank the reviewer for spotting this omission, we have added the following citations as reference of the impact of secondary modifications on tRNA and tRNA-fragment detection (Kimura Nat Chem Biol 2020 and Padhiar Trends Genet 2024).

9. Additionally, a analysis looking into the ratio between full length tRNA transcripts and their tRFs derived from these transcripts would be very interesting here to see whether the expression levels of tRFs corresponds with the specific full length tRNA for all its fragments or just specific fragments remain.

We agree with the reviewer that it would be interesting to systematically assess whether the levels of the tRFs are directly proportional to that of their full-length parental tRNA. Considering the 62-63nt sequence of mt-tRNA^{Ser(GCT)} as the reference full-length form and all shorter reads mapping to this tRNA as derived fragments, we found that, as expected, the highest normalized counts

corresponded to the full-length form. The abundance of the smaller derived fragments ranged between 0.039% to 60% of the parental tRNA counts, with small 5'tRFs (18-19 nt) being the most abundant. Thus, some tRFs appear to be selectively retained compared to others and are not merely the result of full-length tRNA degradation. However, it should be emphasized that the protocol of small RNA-seq used in our study was not optimized for the detection of longer RNA species and many full-length tRNAs were only barely detected. Moreover, the efficiency of detection may vary between isoacceptors, depending on their length and secondary modifications, making this kind of analysis biased and not reliable. Therefore, it was not possible to systematically analyze the ratio between full-length tRNAs and their derived fragments. The need of a specific tRNA profiling is highlighted in [lines 736-737](#).

10. "Interestingly, several mitochondrially-encoded tRFs were upregulated in 22-month-old β -cells" – give precise numbers of tRFs

We have now added in the text the precise numbers of mt-tRF modulated in our datasets ([lines 423-425](#)).

11. In line 314 should be an explanation why these specific miRNAs and tRFs were picked for validation experiments. Was there a specific expression threshold or FC cut off employed?

We thank the reviewer for pointing out this missing information. We now clarified in the manuscript ([lines 355-358](#)) that the miRNAs were selected for validation based on their demonstrated roles in the most relevant pathways modulated in aging: inflammation (miR-146-5p); aging and cell proliferation (miR-181b-5p) and insulin sensitivity and glucose homeostasis (miR-143-3p and miR-145-5p). In addition, miR-34a-5p was included given its well-established association with aging across multiple tissues. The tRFs were selected based on their previously reported or potential roles in beta cell homeostasis and their potential link with pancreatic islet low grade chronic inflammation ([see lines 472-477](#)).

12. Why were 7/8 months old animals chosen for the analysis of senescent cells? There is no reference for this age in the initial data. For this mouse cohort the sex of the mice is missing.

There should also be an indication how many cells were sorted for these two populations. And a comparison for the sorted Beta-cells at this age without the sorting into subgroups to enable and understanding of the contribution of the different subpopulations to the entire population.

Although the number of senescent beta-cells is known to increase with age (Aguayo 2017), our aim here was to generate a dataset of senescent (vs non-senescent) beta-cells that would be independent of aging per se. To this end, we applied the same experimental setting as previously published for transcriptomic sequencing, namely fluorescence-activated cell sorting based on β -gal activity to obtain senescent (β -gal+) vs non-senescent (β -gal-) beta-cell populations from adult male retired breeders of 7-8 months.

Information on mouse sex and the number of FACS-sorted cells has been included in Suppl. Table 3.

13. In line 349 and following the wording induced is misleading and should be replaced by e.g. upregulated.

We agree with the reviewer's comment. As suggested, when referring to an increase in sncRNA levels compared to a control condition, the wording "induced" was replaced by "up-regulated" throughout the manuscript.

14. What is the overlap between deregulated tRFs in the senescent cells and aging?

The overlap with the aging dataset and with previously described data in literature has been added in the Results section [lines 525-527](#). The stress-associated 5'tRF^{Gly(GCC)} that is upregulated in aged β -cells is slightly down-regulated in β -Gal⁺ β -cells (**Figure 4.f**). Another stress-associated 5'tRF from tRNA^{Glu(CTC)} that we recently showed to be involved in T2D pathogenesis and in the modulation of β -cell gene expression was also slightly reduced, while the levels of mt-tRFs were not affected in β -Gal⁺ β -cells (**Figure 4.f**).

15. For the characterization of the cytokine treated islet cells a staining with Beta-Gal, which was previously used to identify senescent cells is missing. There should be a validation with either this or with EdU staining to confirm senescence induction in these cells.

We thank the reviewer for this suggestion. Senescence is characterized by an ensemble of traits that develop through a multi-stage process, encompassing structural changes such as an enlarged lysosomal compartment, the activation of distinct signaling cascades, and the acquisition of a senescence-associated secretory phenotype (SASP), including increased β -galactosidase (β -gal) staining. Since these events occur across different time frames, they are not necessarily detectable at all stages of senescence.

We identified several senescence-associated traits in mouse islets following a wash-out period after cytokine treatment. These included *Cdkn1a* (p21^{Cis}), a well-established marker of cell cycle arrest mediated by the p53–p21 axis; *Il6* and *Serpine1*, as components of the SASP; and *Bambi*, a β -cell aging marker known to interfere with TGF- β and BMP signaling. β -gal staining was also performed; however, we failed to observe an increase in β -gal-positive cells.

Taken together, these findings suggest that our *in vitro* model can recapitulate the chronic low-grade inflammation associated with aging and senescence, but does not fully replicate the complex senescent phenotype that is established over months to years in β -cells. This is now mentioned in [lines 752-761](#).

16. Line 537: There is no through analysis of the piRNA data set in the current version of the paper. This should be included with figures and statistics in the results if the authors claim to a detailed analysis of piRNAs.

We thank the reviewer for this observation. We have now added volcano plots showing the statistical characterization of differentially expressed piRNAs in our datasets (**Suppl. Figure 3.c-d**). This analysis is described in the Results section [lines 401-407](#).

17. Overall, a nice and interesting study however since only male mice were used in the entire study not representative for population top hits and claims need to be validated in a female population.

We are grateful to the reviewer for highlighting the importance of validating our findings in female animals. To address this point, we performed additional analysis in C57BL/6 female mice aged in our institutional facilities. Although mice aged of 12-months were available and suitable for analysis, tumors affecting the liver, intestine or the digestive tract were detected in 8 out 10 female mice aged 22 months, which had therefore to be excluded from our study. Notably, RRHO analysis revealed a significant overlap between the aging miRNA signature identified in male mice and that observed in the female cohort. Furthermore, targeted validations of candidate miRNAs and tRFs confirmed the observations made in 12-month-old male mice ([lines 347-372](#) and [lines 428-437](#) **Suppl. Figure 2.c-d** and **Suppl. Figure 4.c-f**).

Reviewer #3 (Remarks to the Author):

This manuscript presents a comprehensive profiling of small non-coding RNAs (sncRNAs) in pancreatic β -cells and islet macrophages during aging. The authors perform small RNA sequencing on FACS-sorted β -cells and islet macrophages (iMACs) from 3-, 12-, and 22-month-old male C57BL/6J mice and further analyze senescent (SA- β -gal⁺) β -cells as well as cytokine-treated islets. They report age-associated remodeling of several sncRNA classes, including miRNAs, isomiRs, piRNAs and tRNA-derived fragments (tRFs), and propose that aging is associated with an inflammatory miRNA signature in β -cells, an induction of mitochondrial tRFs, and a shift toward an anti-inflammatory or tissue-repair phenotype in aged islet macrophages. The dataset is potentially valuable and the topic is timely, as the role of regulatory RNAs in metabolic aging and β -cell dysfunction has received increasing attention in recent years. Profiling sncRNA dynamics in defined islet cell populations across aging could provide an important resource for the field. Nevertheless, several aspects of the study require clarification or additional experimental support before the conclusions can be considered robust. In its current form the work remains largely descriptive, and several interpretations extend beyond the evidence presented.

Overall, this study provides an interesting dataset describing sncRNA remodeling in aging islet cells and could represent a useful resource for the field. However, clearer reporting of the experimental design, additional technical validation of key RNA species, and stronger functional evidence linking these RNAs to β -cell physiology would substantially strengthen the manuscript. With revisions as indicated, and a more cautious interpretation of the results, the work could make a valuable contribution to our understanding of regulatory RNA networks in islet biology and metabolic aging.

We are grateful to the reviewer for the overall positive evaluation and for providing critical comments to improve our study.

Major.

1. Experimental design and statistical reporting. The authors should clearly report the exact number of independent RNA-seq libraries and biological replicates used for each dataset, including the samples used for qPCR validation. Please clarify these numbers both in the Methods section and directly in the figure legends so that the robustness of the analyses can be assessed.

Details regarding experimental replication and the number of independent experiments have been included in the figure legends. The definition of independent experiment has been added in line 143-145 and for full clarity we added **Suppl. Tables 1-3**, describing the pooling strategy and FACS sorting outputs.

The authors should also justify the differential expression criteria used in the study (fold change >1.5 and FDR <0.1), which appear relatively permissive given the large number of transcripts tested across multiple sncRNA classes.

We now provide numbers of differentially expressed sncRNAs using more stringent thresholds of FDR<0.05 and FC>2.0. Applying the more stringent thresholds did not affect the overall observed effects on pathway enrichment. We also explicitly explain in the text that less stringent thresholds were used for the metanalysis approach in order to capture potential trends in the dynamics of aging islets.

In addition, report the total number of tested transcripts per RNA category (miRNAs, tRFs, piRNAs, etc.) and the number retained after statistical filtering.

We have now updated the result section to include details on the number of sncRNAs detected from the annotation process and passing statistical thresholds upon differential expression analysis. Lines 317, 353, 376, 404, 411, 430, 505, 620.

In several cases the qPCR validation experiments show only trends rather than statistically significant changes, for example for miR-34a-5p, mitochondrial 12S/16S fragments, and several tRFs (Fig. 3 and Supplementary Fig. 3; $p \approx 0.06-0.1$). Similarly, temper the associated conclusions and clearly acknowledge when validation experiments do not reach statistical significance.

We thank the reviewer for pointing out this issue. We have now rephrased our results when referred to non-significant data.

2. From descriptive to mechanism in β -cells. A broader limitation of the manuscript is the lack of functional evidence linking the identified RNAs to β -cell aging or dysfunction. Because the manuscript frequently interprets these RNA changes in terms of β -cell function, inflammation, or metabolic decline, the authors need to provide additional phenotypic characterization of the same cohorts of mice used for sequencing. Specifically, they should measure and report functional parameters such as glucose-stimulated insulin secretion, basal insulin secretion, insulin content, proliferation markers (e.g. Ki67), and classical senescence markers including p16, p21 or SA- β -gal activity. These phenotypes should be correlated where possible with the expression of key RNAs identified in the study. Previous studies have shown that aging β -cells display increased expression of senescence regulators such as p16Ink4a and p21, reduced proliferative capacity, and altered mitochondrial metabolism (Aguayo-Mazzucato et al., Cell Metabolism, 2017; Helman et al., Nature Communications, 2016).

As noted by the reviewer, several previous studies have characterized islet function in aged mouse models. In general, beta-cell function tends to decline with age, leading to increased basal insulin secretion and a reduced capacity to compensate for the insulin resistance of peripheral tissues (Aguayo 2020 review).

In the present study, we elected to perform RNAseq on FACS-sorted beta-cells and macrophages rather than whole islets, in order to specifically characterize sncRNA changes in these individual cell types. To maximize the yield of sorted cells, islets were allowed a short recovery of 2 hours in complete medium before dissociation and FACS-sorting of beta cells and iMACs. As now shown in Suppl. Table 1, islets of several mice were pooled to obtain sufficient material for sequencing. The number of cells recovered after FACS is also listed in Suppl. Tables 1-2. While this approach improved the quality and quantity of sorted cells and may temper the influence of individual metabolic variability, it limited the possibility of conducting ex vivo experiments on isolated islets. This limitation is now explicitly acknowledged in the new limitations paragraph of the revised manuscript (lines 777-778).

3. The manuscript highlights several miRNAs and tRFs as potential regulators of β -cell “inflammaging,” including miR-146a-5p, miR-142-3p/5p, miR-181a/b, miR-143/145 and tRFs such as 5'tRF-Gly(GCC) or mitochondrial fragments derived from tRNA-Leu and tRNA-Ser. To support these claims, perform gain- or loss-of-function experiments for at least a subset of these candidates in primary islets or β -cell models. The authors need to assess relevant functional outcomes such as insulin secretion, apoptosis, or levels of senescence markers following perturbation of these RNAs. Previous work has demonstrated that individual miRNAs can profoundly influence β -cell biology. For example, miR-375 regulates insulin secretion and β -cell mass (Poy et al., Nature, 2004), and disruption of miRNA processing in β -cells leads to severe defects in β -cell function and survival (Latreille et al., Genes & Development, 2014), plus many

studies from the authors own laboratory. I recommend using similar approaches to determine whether any of the candidate RNAs, as identified in this study, directly contribute to aging phenotypes.

We thank the reviewer and agree that gain- and loss-of-function studies represent an important step toward establishing causal roles for age-associated small RNAs in β -cell biology. As mentioned by the reviewer, our group and others have previously characterized the function of several age-associated miRNAs; these functional studies are summarized in Suppl. Data_6, with an indication of the functional effects of specific miRNAs. Although the characterization of tRF function in β -cell homeostasis is a novel area of research, we have previously identified the involvement of some cytosolic and mitochondrial tRFs in β -cell maturation and dysfunction in type 1 and type 2 diabetes. Notably, some of these tRFs were found to be modulated during aging. A more detailed description has been included in the Results ([lines 419-423](#)) and Discussion ([lines 710-729](#)) sections. Moreover, we have performed additional gain- and loss-of-function experiments for miRNAs and mitochondrial tRFs deregulated during aging; these results are presented in a new paragraph of the Results section ([lines 587-610](#)) and in **Suppl. Figures 6 and 7**. While overexpression of two miRNA combinations including miRNAs belonging to a cluster (miR-143/145) and two different families (miR-10 and miR-126) do not significantly affect cell viability or beta-cell function, we validated the role of mt-tRFs as modulators of beta-cell insulin secretion and mitochondrial function.

4. tRF detection and mitochondrial interpretation. The analysis of tRNA-derived fragments requires further clarification. Please provide additional information on the detection and classification of tRFs, including the length distribution of detected fragments, the relative representation of mitochondrial versus nuclear tRFs, and the number of fragments passing statistical thresholds. Because the sequencing strategy did not include removal of tRNA modifications and the mapping strategy appears to require perfect sequence matches, please discuss the potential biases introduced by these methodological choices.

We have now provided additional details describing the tRF datasets, including mt- / cyto-tRF and length representation ([Lines 410-415 and 431-433](#) and Suppl. Figure 4.a-b). The implications of including steps to remove tRNA modifications have been now extensively discussed in [lines 716-719](#) and references were added (Kimura Nat Chem Biol 2020 and Padhiar Trends Genet 2024). Notably, we provided novel small RNA-seq data of islet cells obtained from female mice, including a step of modification removal. Interestingly the overall upregulation of mitochondrial tRFs was detected also in β -cells from female aged mice.

Independently validate several representative fragments (for example 5'tRF-Gly(GCC), mt-5'tRF-Ser(GCT), or mt-itRF-Leu(TAA)) using orthogonal approaches such as Northern blotting or ligation-based qPCR. Until such validation is provided, moderate the claim that mitochondrial tRFs are globally induced during aging.

We acknowledge that validation using orthogonal approaches would be ideal to confirm our observations. However, the limited amount of starting material from FACS-sorted cells precluded the use of these techniques in this study. Thus, we have moderated our claims accordingly and discussed this limitation in [lines 784-786](#).

5. Inflammation vs aging causality. The cytokine treatment experiments are interpreted as a model of aging-related inflammation, yet the relationship between these conditions is not fully demonstrated. Acute cytokine exposure may reflect stress responses rather than the chronic inflammatory environment associated with aging. Please quantify the overlap between cytokine-

regulated sncRNAs and those altered in aged or senescent β -cells. Present this analysis explicitly, for example using overlap statistics or enrichment analyses. If the cytokine treatment is intended to mimic aspects of aging or senescence, then please provide functional measurements such as insulin secretion or senescence marker expression under these conditions.

We thank the reviewer for highlighting the need of further investigation. As noted, the inflammatory profile established during aging likely reflects low-grade chronic inflammation and probably differs from the acute stress response induced by direct exposure to elevated cytokine concentrations. To explore this further, we performed a Rank-Rank Hypergeometric Overlap (RRHO) analysis comparing differentially expressed miRNAs and isomiRs in aging (22 months vs young) and in response to cytokine treatment (cyto vs ctr). Although significant correlations were found among entries modulated in the same direction under both conditions, this analysis clearly highlighted differences between the iso/miRNome signatures defined by the 2 conditions. Applying threshold of FDR <0.1, we identified miRNAs and isomiRs detected and modulated in both datasets. Among these, 8 small RNAs were modulated in the same direction, including miR-146-5p and miR-143-3p. Fifteen miR/isomiR molecules were downregulated by cytokines but upregulated in aging, including miR-409-3p, previously reported to be downregulated in plasma from T1D patients and upregulated in T2D patients. Finally, 10 miR/isomiRs were upregulated by cytokines but downregulated during aging, including miR-21a-5p, previously associated with cytokine-induced apoptosis. These results are shown in [lines 388-400](#), **Figure 2.c-d**.

We have also clarified more explicitly that the cytokine-plus-recovery model used to mimic an aged phenotype in vitro was designed to induce a sustained inflammatory environment while minimizing the confounding effects of acute stress responses. Our findings show that senescence markers are induced exclusively under chronic cytokine-plus-recovery condition, whereas other gene and miRNA markers are induced during the acute response and remain elevated following recovery.

6. iMAC phenotype. The proposed phenotype of aged islet macrophages also requires additional support. The authors suggest that aged iMACs may adopt an anti-inflammatory or tissue-repair phenotype based largely on miRNA expression patterns. Complementary expression analysis of classical macrophage activation markers (including Tnf, Il1b, Il6 and Nos2 for inflammatory states and Il10, Arg1 or Mrc1 for repair-associated states) would provide a more direct assessment of macrophage polarization. If feasible, simple ex vivo assays measuring phagocytic or efferocytic activity could further strengthen the proposed interpretation.

We thank the reviewer and acknowledge that a more detailed characterization of aging iMACs would be of considerable interest to the field. However, the limited amount of material recovered from FACS-sorted iMACs in this study did not allow for additional phenotypic investigation. These limitations are discussed in [lines 764-767](#).

7. Figure-specific aspects. In the differential expression analyses (Fig. 1–2), the authors should clearly indicate the statistical thresholds used, the number of tested transcripts per RNA class, and the number of significant hits after correction for multiple testing. In the validation experiments (Fig. 3 and Supplementary Fig. 3), some of the reported changes appear to approach but not reach statistical significance, and the corresponding conclusions should be adjusted accordingly.

We have now included details of statistical thresholds in the figure legends.

In the comparison between β -cells and iMACs (Fig. 4), it would also be useful to clarify how normalization was performed when comparing two cell types with very different baseline RNA expression profiles.

We would like to clarify that the present study did not include comparisons between iMACs and β -cells, and that all differential expression analyses were performed within the same sequencing datasets. Comparisons are based exclusively on Log₂ fold changes derived from differential expression analysis. Figure 4 refers to the differential expression analysis of β -gal positive versus β -gal negative β -cells isolated from the same islet preparation (Figure 4.a), using four independent islet preparations (as now stated in the figure legend and detailed in Suppl. Table 3). The Log₂ fold changes from the aged (12 or 22m) vs young (3m) and β -gal+ vs β -gal- comparisons were then plotted to compare miRNA modulation across the different datasets. The description of these results has been rephrased for clarity in [lines 502-505](#).

Finally, the cytokine treatment experiment (Fig. 5) would benefit from a quantitative comparison with the aging datasets to better establish the relationship between inflammatory stimulation and age-associated RNA remodeling.

We thank the reviewer for this observation. As in this study small RNA-seq of islets undergoing transient inflammatory treatment followed by recovery was not performed, we do not dispose of the dataset to perform an unbiased quantitative comparison.

8. Scope and Limitation. Finally, several limitations of the study should be more explicitly acknowledged in the Discussion. The work is restricted to male C57BL/6J mice, metabolic phenotyping of the animals is limited (body weight and fed glycemia only), and most conclusions are based on correlative analyses. In addition, the Discussion occasionally overstates the potential translational implications of the findings, particularly the suggestion that these RNA signatures might help predict individuals who will age metabolically healthy versus those who will develop metabolic disease. Given the current data, such claims appear premature.

We agree with the reviewer on the need to explicitly acknowledge the study's limitations. We decided to add a closure paragraph dedicated to the limitations and perspectives. We are confident that this structure will transparently reflect the still existing gaps but also the open opportunities of future research.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank all the reviewers for their positive comments on the revised version of our manuscript. We have addressed the remaining points raised during the second round of review, both in the revised manuscript and in our detailed responses to the reviewers' comments.

Reviewer #1 (Remarks to the Author):

I thank the authors for their thorough and thoughtful responses to my comments. I appreciate the extensive revisions made to the manuscript, and I believe that my major concerns have been satisfactorily addressed. The additional analyses, clarifications, and discussion have substantially strengthened the manuscript.

I only have a few minor remarks that should be addressed before publication:

Figures:

The figure legend for panels 2c,d appears to be missing.

[Thank you for spotting this omission, the legend has now been revised.](#)

Figure 4: The symbols in panels 4a and 4e are not displayed correctly.

[We have corrected this issue.](#)

Figure labeling: The labels in several panels (e.g., Figures 2d and 4b) are very small and difficult to read. Increasing the font size would improve readability.

[We have now increased the size of the plots and text in figures to improve readability.](#)

Figure 5b: It would be helpful to provide representative images.

[Representative images have been provided in Supplemental material.](#)

Supplementary Figure 1a,b: The labeling appears to be slightly misaligned and could be adjusted for improved clarity.

[The labels have now been adjusted.](#)

Overall, I congratulate the authors on this comprehensive study and thank them for carefully addressing the reviewers' comments.

[We are grateful to the reviewer for his positive evaluation of our work.](#)

Reviewer #2 (Remarks to the Author):

All comments were addressed. Good work.

[We thank the reviewer for his positive feedback.](#)

Reviewer #3 (Remarks to the Author):

The authors have addressed many of the concerns raised. The manuscript is certainly improved, and the addition of functional experiments, particularly regarding mt-tRFs, provides valuable insights. However, some critical points remain that require clarification, or more cautious interpretation, before the work can be considered robust.

We thank the reviewer for the positive evaluation of our revision work. We have now addressed all the additional points raised in the second revision.

Major points

1. Please clearly distinguish the acute 24-hour cytokine small-RNA sequencing dataset from the separate cytokine-plus-washout model used for the functional experiments in Figure 5. The number of independent biological replicates for the 24-hour sequencing dataset should be stated explicitly in the Methods and Figure 2 legend, since the RRHO analysis depends on it.

We thank the reviewer for these insightful comments. We have modified accordingly the Methods and Results sections, and the figure legends. The number of independent biological replicates for acute cytokine treatment (n=3) and ageing datasets (n=4) is now clearly indicated.

Please also clarify in the Manuscript that the RRHO analysis evaluates overlap between acute cytokine and aging-associated sncRNA changes, whereas the washout experiment provides targeted phenotypic validation and does not recapitulate the complete aging sncRNA profile.

This is now clearly stated in the result session (see lines: 177-178 and 318-320).

2. The new mt-tRF loss-of-function experiments are strong and really useful, but they were performed in young islet cells and MIN6B1 cells, not in the aged β -cells where the RNA changes were identified. Please either provide direct phenotyping of the aged cohorts or clarify throughout the manuscript that the age-associated RNA changes remain correlative. Revise the interpretation from “mt-tRFs maintain mitochondrial function during aging” to “mt-tRF perturbation can influence mitochondrial function and insulin secretion in the tested cellular models.”

We have clarified the use of young islet cells (line 334) and revised the conclusive sentence in the result section accordingly (see lines: 347-348). Limitations of the interpretation of our results are also clearly mentioned in the discussion.

3. The manuscript states that no significant age-dependent changes were detected at 12 months, but then notes that all detected full-length tRNAs were “upregulated” at 22 months. Please clarify whether “upregulated” means statistically significant or simply higher relative sequencing counts. Since this method cannot reliably measure absolute RNA levels or the exact relationship between full-length tRNAs and their fragments, please use more cautious wording such as “higher relative sequencing abundance” or “age-associated enrichment.”

Significant changes of sequences corresponding to full length mitochondrial tRNAs were defined by FDR p-value < 0.05, Fold Change > 2; this is now clearly stated. Following these criteria, we found that all the detected (passing count number filtering) full length mitochondrial tRNAs were significantly upregulated in beta cells from 22-month-old male mice compared to 3-month-old. Changes were not significant in 12-month-old male or female mice. This is now better highlighted in the text. We have also rephrased the sentence using “relative abundance” as suggested by the reviewer (see lines: 243-247).

4. The manuscript continues to infer an “anti-inflammatory” or “tissue-repair” phenotype for aged iMACs based primarily on miRNA patterns, without direct measurements of macrophage activation markers or functional assays like phagocytosis. While the added limitation statement is helpful, the Abstract and Results remain strong in their inferences. Please either add direct marker/functional data or revise the language to state: “The aged iMAC miRNA profile was consistent with a possible anti-

inflammatory or homeostatic remodeling, but this phenotype was not directly established."

We have revised the language to further ensure that our interpretations are framed as hypotheses or suggestions based on the differential expression miRNAs that have been previously associated with a specific macrophage polarization state, rather than as direct evidence of a functional phenotype (see lines 363-364 and 479-481).

Minor Text Corrections:

- Correct "FAC-sorted" to "FACS-sorted" throughout;
- Correct "reduced of Sirt1" to "reduced Sirt1" or "reduced expression of Sirt1";
- Ensure the term "aging-or senescence-like" includes proper spacing or hyphenation;
- Harmonize nomenclature for "miR-181a-5p" and "miR-181b-5p";
- Minor figure-legend labeling problem in Figure 2;
- Please consistently use absolute fold-change notation to avoid ambiguity;
- Please clarify the significance threshold in figure legends to ensure p-values between 0.05 and 0.1 are visually and textually distinguished from statistically significant values ($p < 0.05$);

We thank the reviewer for highlighting these minor corrections that have been incorporated into the revised version of the manuscript.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.