

Suppressor tRNAs restore functionally enhanced tumour suppressor protein to induce tumour regression

Corresponding Author: Dr Qing Xia

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)

In this manuscript, Kong et al. present a study on the use of suppressor tRNAs (sup-tRNAs) to restore expression of tumor suppressor genes harboring nonsense mutations, with a primary focus on TP53. They demonstrate that sup-tRNA-mediated rescue of TP53 suppresses tumor cell proliferation in xenograft mouse models, highlighting a potential therapeutic avenue for cancer treatment. They also describe the use of computational modeling to optimize sup-tRNAs for enhanced p53 activity through incorporating non-wildtype amino acid residues.

Overall, the manuscript presents an interesting and potentially impactful approach to cancer treatment. However, several key conclusions are not sufficiently supported by experimental evidence, and several key experiments lack scientific rigor. Using stop codon readthrough to rescue tumor suppressor gene expression has been reported in several publications (as the authors correctly referenced). Although this manuscript shows progress in using sup-tRNA, the significance of the work is limited as it is unclear whether the in vivo sup-tRNA delivery approach is translational. Labeling in many figures lacks clarity. Major concerns:

1. In lines 148–150 and 222–224, the authors suggest that specific sup-tRNAs (e.g., Gln or Arg) restore TP53 function and that certain substitutions (e.g., S121F, Q144R, H214Q) enhance TP53 activity. However, engineering tRNA anticodons or body sequences may lead to tRNA misacylation (PMID: 35322228; PMID: 39673265). The actual amino acids incorporated by these sup-tRNAs must be experimentally identified, for example, through mass spec-based assays. Additionally, functional assays should directly compare TP53 variants bearing these missense mutations to wildtype TP53 to validate the claimed enhancements.
2. In the evaluation of tumor cell proliferation in vitro and in vivo, it is unclear whether the observed inhibitory effects are due to TP53 restoration or potential sup-tRNA toxicity. Swapping sup-tRNAs between tumor cells with UAG and UGA nonsense mutations could help clarify this.
3. In vivo data demonstrating enhanced TP53 activity restoration by specific sup-tRNAs (as predicted by computational modeling) are lacking.
4. In Fig S4b, S4c 4e and 4f, the functional restoration results are not directly comparable across different sup-tRNAs and nonsense mutations, as readthrough efficiency can vary depending on the specific sup-tRNA, the type of premature stop codon, and the surrounding sequence context. Normalization of functional restoration (i.e., mCherry) to TP53 protein restoration levels (i.e., EGFP) may help draw clearer conclusions. Additionally, EGFP expression data are missing from Figures S4b and S4c.
5. In Fig 2d, the claim that “sup-tRNAGln outperformed sup-tRNA^{Trp} in functional restoration of p53-W146X mutant” is not convincingly supported by the data presented. A more direct comparative analysis is needed.
6. According to the cited reference (PMID: 36433934), 5-Fluorouridine seemed restore TP53 levels in vivo to a higher level than reported in this study. Please discuss the advantages or potential improvements of the sup-tRNA strategy in comparison.
7. In Fig 3a-c, the immunostaining for CDKN1A in CRC cell lines appears suboptimal, and the conclusions are not backed by quantitative analysis. Replicates and quantification are needed to substantiate the results.
8. The use of EGFP and mCherry fluorescence area to assess TP53 expression and function may lead to quantification bias. Quantifying fluorescence intensity or performing Western blotting may offer more accurate measurement of protein expression levels.
9. In Fig 5f and 5k, the criteria used to define survival in the Kaplan–Meier curves should be clearly described.
10. The endpoints for the two tumor cell line xenograft models differ (Day 22 and Day 50), but no rationale is provided in the manuscript.

11. In Fig 5l and 5m, it is unclear what each dot in the bar graphs represents. Additionally, the expression level of CDKN1A appears inconsistent with TP53 levels and requires explanation.

12. The in vivo experiments lack crucial information about model generation, sup-tRNA delivery and G418 administration, dosages, and treatment regimens. Additionally, systemic delivery may offer greater clinical relevance than the intra-tumoral injection used in this study.

13. Figures should be rearranged to make all labels and drawings clearly legible.

Minor concerns:

- Figures should be called out in order.

- Fig 1a, 1b and S1: Their interpretation is unclear from the current text. Consider expanding the explanation in the Results section rather than in the Introduction.

- Fig 1c and S3a: Use of similar colors across multiple amino acids may cause confusion.

- Fig 1e: Details of the dual-luciferase reporter assay method and reporter construct design are missing.

- Please provide a schematic of the sup-tRNA construct design and include the complete sup-tRNA sequences.

- Fig 1g and h: The specific mutations (TAG, TAA or TGA) and their corresponding sup-tRNAs are not indicated.

- Fig 1h: The readthrough level of "PTEN PTC Q17X" is higher than sup-tRNA treatment and is essentially the same as "PTEN WT", which is counterintuitive and needs clarification.

- Fig 1h: PylRS-tRNA is shown but not described in the manuscript.

- Fig S3: This figure (and some other figures) is not referenced in the manuscript.

- Line 259 and Fig 3a: The nonsense mutations in Calu-6 and Caco-2 cell lines are not clearly described in the text (TAG, TAA, or TGA?), and the western blot images are not clearly labeled with corresponding cell lines.

- Fig 3b: Two images are labeled "CDKN1A Arg-TCT-3-1 TGA," which appears to be a labeling error.

- Fig 3g: It is unclear whether the enriched KEGG pathways are upregulated or downregulated.

Reviewer #2

(Remarks to the Author)

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

Reviewer #3

(Remarks to the Author)

In this paper, Kong et al. develop a platform to restore tumor suppressor function using engineered suppressor tRNAs (sup-tRNAs) that enable codon-specific readthrough of nonsense mutations. They focus on TP53, demonstrating that sup-tRNAs can restore both protein expression and transcriptional function in a dual-reporter assay (Figure 2a). They also show that some amino acid substitutions restore full-length p53 but fail to rescue activity (e.g., Y234Q, G266R), which they attempt to explain through structural modeling (Fig. 2e) and predictive scoring (Fig. 2f). Restoration is validated in endogenous mutant cell lines (Calu-6, Caco-2) using Western blot, qPCR, and transcriptional profiling (Fig. 3), and sup-tRNA therapy significantly reduces tumor growth in xenografts (Fig. 5). The "break-bad" strategy, using non-native amino acids to produce gain-of-function p53 variants, is a super clever angle that could push this approach beyond simple restoration.

Overall, I really like this study, and the core idea and data are quite compelling and relevant. I just have a few additions and clarifications to elevate the manuscript further:

1. The structural claims around deleterious substitutions like Y234Q and G266R are based on static models. I'd really like to see the authors quantify how those substitutions alter local flexibility or overall stability during the MD simulations. Just showing RMSD and per-residue RMSF across the DBD would make the modeling section far more convincing than visual inspection alone.

2. Nowhere do the authors directly confirm that the intended amino acid is inserted at the site of readthrough. That's a big gap. A straightforward LC-MS/MS experiment on p53 pulled down from Calu-6 or Caco-2 cells could close this loop? It would directly validate the specificity of the sup-tRNA and rule out unintended wobble pairing or misincorporation.

3. There's no check on sup-tRNA expression across constructs. That makes it hard to interpret read-through efficiency comparisons in Fig. 2b or Fig. 3a. It wouldn't take much (RT-qPCR or even Northern blot) for the authors to verify whether the differences they're seeing are biological or just a function of expression variability.

4. The computational selection framework (Fig. 2f) is presented as a black box. It would help to see even a small benchmark: say, how well the model predicted the best-performing sup-tRNA for 10 p53 nonsense variants, compared to what actually worked. Without that, it's hard to judge if the model meaningfully informed tRNA design.

5. The in vivo work relies on intratumoral injection, which is perfectly fine for proof-of-concept. I'd love to see the authors test an LNP-based or EV-based delivery system in vitro, just something to show that sup-tRNAs can be delivered more broadly than with direct injection. That kind of experiment would make this feel closer to translational reality. But definitely not necessary.

This is a well-designed and exciting paper, and with a few additions like these, it could be a really definitive statement on the

therapeutic use of engineered tRNAs! Overall, upon these minor changes, I will recommend acceptance.

Reviewer #4

(Remarks to the Author)

"Restoration of tumor suppressor protein with enhanced activity using engineered tRNAs to induce tumor suppression", by Kong et al.

The manuscript describes the generation of a series of sup-tRNAs for several p53 non-sense mutations and their effects on inducing p53 expression and the biological consequences thereof.

Overall, the concept of promoting readthrough at stop codons using various methods, including drugs like G418 has been previously shown for many genes. These further include the tumor suppressors p53 and PTEN. Moreover, the use of sup-tRNAs to overcome stop codons have also been well established (Ref 25 and 26 in this paper). As such, the conceptual novelty of the present findings is significantly reduced, as the authors have now tested a set of specific sup-tRNA for several p53 non-sense mutations to demonstrate readthrough.

The quality of the work needs significant improvement. Many controls are lacking – as an example, there is no demonstration that the sup-tRNAs are indeed in the tumor, or other organs, in the in vivo study. The global RNA seq analyses only used the cells with mutant p53, and do not include p53 null cells. This will be an important control to evaluate the effects (non-specific) of the sup-tRNAs. Moreover, demonstration that the sup-tRNAs that are generated for the indicated p53 mutations can or cannot work on others genes in the genome needs to be clarified (globally). This is crucial control to exclude non-specific effects, that are associated with G418.

The quality of the presentation of the data can be significantly improved, and in its current state, posed a challenge in the review process. Firstly, the fonts and figures are minute, even after enlarging on the screen. Secondly, very little details are given on the procedures. An example (among many) is that there is no information on how the tRNAs were delivered in vivo. There is a sentence in the M&M that the sup-tRNAs were delivered by dendrimer nanoparticles. No reference or details are given.

The references to the figures in the text are not in order and some are missing. For e.g. after Fig 2, the authors refer to Fig 4, and then come back to Fig 3. More thought in the organisation of the manuscript is needed.

Language can be significantly improved. An example (among many) is on the M&M on xenografts: ".....Caco-2 cells were injected into the substance of each mouse..." It is unclear what this means, and the M&M does not provide reliable information on how this is done. In the Intro section, the sentences on the driver ability are unclear to understand what the authors are trying to imply.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revision has addressed many concerns from the previous review with additional experiments and discussion. Data presentation is also significantly improved. However, the current version still suffers from several issues.

Major concerns:

1. The premise of using in silico predication to design specific suppressor tRNAs that introduce non-WT, activity-enhancing residues is a key claim, but it is not sufficiently supported by the data. Many experiments comparing suppressor tRNAs that introduce WT or non-WT residues only have p53 function data, but lack protein amount data showing how much p53 protein is restored (Fig. 4, Supplementary Fig. 5). P53 protein restoration should be quantified by western blot to see whether the amount of protein restoration, rather than the residue identity, is the major determinant for functional restoration.

2. The new data presented in Fig. 6 is based on a p53-null cell line with a p53-R210* transgene introduced by lentivirus transduction, but not cells with endogenous p53 nonsense mutations as in Fig. 5. Using the transgenic cell line poses a major caveat regarding how much p53 protein can be maximally restored, and whether it is relevant to the level of endogenous p53. For example, a strong promoter, multiple transgene copies, lack of mutant mRNA degradation by NMD can all artificially boost protein restoration and exaggerate efficacy. Therefore, the experiments in Fig. 6 should be repeated using the cell model(s) in Fig. 5.

Minor suggestions:

1. Fig. 1e: why these tRNAs were selected from each family for testing? All glutamate tRNAs failed - please elaborate on this result.
2. New mass spectrometry data is shown, but lacks Methods information.
3. Fig. 2f (and other figures): should "Being" be "Benign"?
4. Line 196: should those tRNAs targeting UAG?
5. Line 256: should it be Supplementary Fig. 8b, not 9b?
6. Again, please make sure figures are called out in order. For example, Supplementary Fig. 18 and 19 now are mentioned before Supplementary Fig. 16 and 17.

Reviewer #2

(Remarks to the Author)

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

Reviewer #3

(Remarks to the Author)

The authors have satisfactorily responded to all of my comments. I recommend acceptance.

Reviewer #5

(Remarks to the Author)

the authors have significantly improved the manuscript organisation, language and Figures. They have performed important additional experiments in accordance with referees criticism, which provide substantial evidence supporting author's conclusions.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed all my concerns.

Reviewer #2

(Remarks to the Author)

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

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Point-by-Point Reply to Reviewers' Comments

We are grateful to the editor and reviewers for their constructive and positive comments, which have greatly helped us improve the quality of the manuscript. We have carefully addressed all the issues raised. Below are our point-by-point responses to the reviewers' comments and questions, with questions in black and responses in blue, respectively.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, Kong et al. present a study on the use of suppressor tRNAs (sup-tRNAs) to restore expression of tumor suppressor genes harboring nonsense mutations, with a primary focus on TP53. They demonstrate that sup-tRNA-mediated rescue of TP53 suppresses tumor cell proliferation in xenograft mouse models, highlighting a potential therapeutic avenue for cancer treatment. They also describe the use of computational modeling to optimize sup-tRNAs for enhanced p53 activity through incorporating non-wildtype amino acid residues.

Overall, the manuscript presents an interesting and potentially impactful approach to cancer treatment. However, several key conclusions are not sufficiently supported by experimental evidence, and several key experiments lack scientific rigor. Using stop codon readthrough to rescue tumor suppressor gene expression has been reported in several publications (as the authors correctly referenced). Although this manuscript shows progress in using sup-tRNA, the significance of the work is limited as it is unclear whether the in vivo sup-tRNA delivery approach is translational. Labeling in many figures lacks clarity.

Response:

We sincerely thank the reviewer for these comments and valuable suggestions. We have comprehensively restructured the manuscript and added a substantial body of new data to strengthen the significance of this work. Furthermore, to validate the translational potential of our in vivo sup-tRNA delivery approach, we performed additional experiments combining sup-tRNA with the FDA-approved chemotherapeutic agent nab-paclitaxel, and found that their combination induced near-complete tumor regression without detectable toxicity (lines 531-576). We have also redrawn all figures and revised the labeling to ensure their clarity and readability.

Major concerns:

1. In lines 148–150 and 222–224, the authors suggest that specific sup-tRNAs (e.g., Gln or Arg) restore TP53 function and that certain substitutions (e.g., S121F, Q144R, H214Q) enhance TP53 activity. However, engineering tRNA anticodons or body sequences may lead to tRNA misacylation (PMID: 35322228; PMID: 39673265). The actual amino acids incorporated by these sup-tRNAs must be experimentally identified, for example, through mass spec-based assays. Additionally, functional assays should directly compare TP53 variants bearing these missense mutations to wildtype TP53 to validate the claimed enhancements.

Response: We are grateful to the reviewer for the valuable suggestion. The actual amino acids incorporated by the sup-tRNAs were verified by mass spectrometry (Supplementary Fig. 3a), and structural simulations confirmed that there were no significant conformational changes in the sup-tRNAs compared with their native tRNA counterparts (Supplementary Fig. 3b, lines 134-139). These results demonstrate that the sup-tRNAs used in this study enable the precise incorporation of specific amino acids at nonsense mutation sites, consistent with previous reports (PMID: 30778053, 39673265). The

p53 variants with enhanced function used in this work have been validated in multiple previous studies (PMID: 15781620, 10449408, 22783376, 24133622), and we thank the reviewer for pointing this out. We have updated the citations for these enhanced p53 variants to strengthen this conclusion.

2. In the evaluation of tumor cell proliferation in vitro and in vivo, it is unclear whether the observed inhibitory effects are due to TP53 restoration or potential sup-tRNA toxicity. Swapping sup-tRNAs between tumor cells with UAG and UGA nonsense mutations could help clarify this.

Response: We sincerely thank the reviewer for pointing out this question. To address this question, we have performed additional in vivo experiments to clarify this issue, and have provided a dedicated section describing these results in the revised manuscript (**Supplementary Figs. 18-20, lines 488-513**). Specifically, in the p53-null tumor model, we found that sup-tRNA administration did not exert any detectable tumor suppressive effects, nor did it induce any treatment-related toxicity. Furthermore, we performed global RNA seq analysis on tumor tissues from this p53-null model following sup-tRNA treatment, and the results confirmed that sup-tRNA exposure did not cause significant genome-wide dysregulation of other genes, nor did it induce non-specific perturbations of the p53 signaling pathway in the absence of mutant p53 (Supplementary Fig. 19).

3. In vivo data demonstrating enhanced TP53 activity restoration by specific sup-tRNAs (as predicted by computational modeling) are lacking.

Response: We thank the reviewer for pointing this out. We acknowledge this limitation and have included a discussion of it in the revised manuscript (**lines 409-417**).

4. In Fig S4b, S4c 4e and 4f, the functional restoration results are not directly comparable across different sup-tRNAs and nonsense mutations, as readthrough efficiency can vary depending on the specific sup-tRNA, the type of premature stop codon, and the surrounding sequence context. Normalization of functional restoration (i.e., mCherry) to TP53 protein restoration levels (i.e., EGFP) may help draw clearer conclusions. Additionally, EGFP expression data are missing from Figures S4b and S4c.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript accordingly (**Supplementary Figs. 5-7**). In the revised manuscript, we have directly compared the readthrough efficiency of different sup-tRNAs targeting the same nonsense mutation. To better evaluate the therapeutic potential of the sup-tRNAs, we performed these assessments in tumor models harboring endogenous *TP53* nonsense mutations rather than in cDNA overexpression systems, and we validated functional restoration via the mCherry reporter assay. We have also revised the text and figures to improve the clarity of the presentation.

5. In Fig 2d, the claim that “sup-tRNA^{Gln} outperformed sup-tRNA^{Trp} in functional restoration of p53-W146X mutant” is not convincingly supported by the data presented. A more direct comparative analysis is needed.

Response: We sincerely thank the reviewer for this valuable suggestion. We agree with the reviewer's comment and have revised it accordingly (**lines 238-244**) .

6. According to the cited reference (PMID: 36433934), 5-Fluorouridine seemed restore TP53 levels in vivo to a higher level than reported in this study. Please discuss the advantages or potential improvements of the sup-tRNA strategy in comparison.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript

accordingly. We established a *TP53* nonsense mutation model and performed *in vivo* evaluations using an approach similar to that described in the cited reference (PMID: 36433934). In this model, sup-tRNA treatment seemed achieve a higher level of p53 restoration than that reported in the aforementioned reference (Fig. 6f, lines 531-576).

7. In Fig 3a-c, the immunostaining for CDKN1A in CRC cell lines appears suboptimal, and the conclusions are not backed by quantitative analysis. Replicates and quantification are needed to substantiate the results.

Response: We thank the reviewer for pointing this out and we have updated the figure accordingly.

8. The use of EGFP and mCherry fluorescence area to assess TP53 expression and function may lead to quantification bias. Quantifying fluorescence intensity or performing Western blotting may offer more accurate measurement of protein expression levels.

Response: We thank the reviewer for this valuable suggestion. We agree with the reviewer's comment and have updated the manuscript accordingly (Figure 2, Supplementary Fig. 4-5, Supplementary Fig. 11).

9. In Fig 5f and 5k, the criteria used to define survival in the Kaplan–Meier curves should be clearly described.

Response: We are grateful to the reviewer for this valuable suggestion. We updated the experimental parameters in the Materials and Methods section (lines 905-906).

10. The endpoints for the two tumor cell line xenograft models differ (Day 22 and Day 50), but no rationale is provided in the manuscript.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the relevant experimental parameters in the Materials and Methods section (lines 903-906). Specifically, due to the heterogeneity across cancer types and cell lines, differences in the tumor growth kinetics between the two xenograft models informed the selection of the respective study endpoints. To further address the potential impact of this heterogeneity, we performed additional experiments in another *in vivo* model to validate the therapeutic efficacy of sup-tRNA (Figure 6, lines 548-568).

11. In Fig 5l and 5m, it is unclear what each dot in the bar graphs represents. Additionally, the expression level of CDKN1A appears inconsistent with TP53 levels and requires explanation.

Response: We thank the reviewer for pointing this out, and we have revised the figure accordingly (Figure 5h-m). The apparent inconsistency between CDKN1A expression and TP53 levels is likely attributable to inter-tumor heterogeneity and individual variability across the animal models. To further support our conclusions, we also measured the mRNA expression level of CDKN1A (Figure 5l-m).

12. The *in vivo* experiments lack crucial information about model generation, sup-tRNA delivery and G418 administration, dosages, and treatment regimens. Additionally, systemic delivery may offer greater clinical relevance than the intra-tumoral injection used in this study.

Response: We sincerely thank the reviewer for this valuable suggestion. We have supplemented the Materials and Methods section with all critical information regarding model generation, sup-tRNA delivery, G418 administration, dosages, and treatment regimens (lines 869-914). We acknowledge the limitations

of intratumoral injection for clinical translation and have included a discussion of this limitation and future perspectives in the Discussion section (lines 620-628). To further enhance the clinical relevance of this work, we evaluated the combination of sup-tRNA with the FDA-approved chemotherapeutic agent nab-paclitaxel, and observed robust therapeutic efficacy with this combination regimen (Figure 6, lines 548-568).

13. Figures should be rearranged to make all labels and drawings clearly legible.

Response: We are grateful to the reviewer for this valuable suggestion. We have redrawn and rearranged all figures to ensure all labels and drawings are clearly legible.

Minor concerns:

- Figures should be called out in order.

Response: We sincerely thank the reviewer for this valuable suggestion. We have reorganized the manuscript and renumbered the figures to ensure that all figures are cited in sequential order in the text.

- Fig 1a ,1b and S1: Their interpretation is unclear from the current text. Consider expanding the explanation in the Results section rather than in the Introduction.

Response: We thank the reviewer for pointing this out, and we have updated the manuscript accordingly. We have redrawn these figures and provided a more detailed explanation of the results in the results section (Figure 1a-b, lines 96-111).

- Fig 1c and S3a: Use of similar colors across multiple amino acids may cause confusion.

Response: We sincerely thank the reviewer for pointing this out. We have revised the figure and labeled the corresponding amino acid for each color to eliminate ambiguity.

-Fig 1e: Details of the dual-luciferase reporter assay method and reporter construct design are missing.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript accordingly (lines 676-683).

- Please provide a schematic of the sup-tRNA construct design and include the complete sup-tRNA sequences.

Response: We thank the reviewer for pointing this out. We have provided the complete sup-tRNA construct design (lines 669-672) and the sequences of all tRNAs in the **Supplementary Table**, with the anticodon of each tRNA highlighted in red.

- Fig 1 g and h: The specific mutations (TAG, TAA or TGA) and their corresponding sup-tRNAs are not indicated.

Response: We are grateful to the reviewer for this valuable suggestion. In this section, all nonsense mutations at native arginine codons have the TGA stop codon, with the corresponding sup-tRNA being tRNA^{Arg}_{UGA}. All nonsense mutations at native glutamine codons have the TAG stop codon, with the corresponding sup-tRNA being tRNA^{Gln}_{UAG}. We have further updated the figure and supplemented this information in the figure legend (lines 90-94).

- Fig 1h: The readthrough level of “PTEN PTC Q17X” is higher than sup-tRNA treatment and is essentially the same as “PTEN WT”, which is counterintuitive and needs clarification.

Response: We thank the reviewer for pointing this out. We attribute this observation to translation reinitiation of the PTEN protein, which occurs because the Q17X nonsense mutation is located at the extreme N-terminus of the protein. This phenomenon was not observed for any other nonsense mutation sites in this study, and we therefore conclude that it is unique to the PTEN Q17X variant.

-Fig 1h: PylRS-tRNA is shown but not described in the manuscript.

Response: We thank the reviewer for pointing this out. We agree with the reviewer's comment and have revised the manuscript accordingly.

-Fig S3: This figure (and some other figures) is not referenced in the manuscript.

Response: We sincerely thank the reviewer for this valuable suggestion. We have updated the manuscript to ensure all supplementary figures are cited in the main text.

-Line 259 and Fig 3a: The nonsense mutations in Calu-6 and Caco-2 cell lines are not clearly described in the text (TAG, TAA, or TGA?), and the western blot images are not clearly labeled with corresponding cell lines.

Response: We thank the reviewer for pointing this out. We have revised the manuscript accordingly (Supplementary Fig. 9e-f, lines 295-299). In the updated results, the western blot images are clearly labeled with the corresponding cell lines (Figure 3a).

-Fig 3b: Two images are labeled “CDKN1A Arg-TCT-3-1 TGA,” which appears to be a labeling error.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript accordingly to correct this labeling error.

-Fig 3g: It is unclear whether the enriched KEGG pathways are upregulated or downregulated.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript accordingly (Figure 3g-h). To further clarify these results, we have included a gene expression analysis of the p53 signaling pathway from the RNA-seq data. In addition, all relevant sequencing data have been deposited in a public database (lines 964-968).

Reviewer #2 (Remarks to the Author):

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Response: We sincerely thank the reviewers for their valuable work and contributions to the peer review process.

Reviewer #3 (Remarks to the Author):

In this paper, Kong et al. develop a platform to restore tumor suppressor function using engineered suppressor tRNAs (sup-tRNAs) that enable codon-specific readthrough of nonsense mutations. They focus on TP53, demonstrating that sup-tRNAs can restore both protein expression and transcriptional function in a dual-reporter assay (Figure 2a). They also show that some amino acid substitutions restore full-length p53 but fail to rescue activity (e.g., Y234Q, G266R), which they attempt to explain through structural modeling (Fig. 2e) and predictive scoring (Fig. 2f). Restoration is validated in endogenous mutant cell lines (Calu-6, Caco-2) using Western blot, qPCR, and transcriptional profiling (Fig. 3), and sup-tRNA therapy significantly reduces tumor growth in xenografts (Fig. 5). The “break-bad” strategy, using non-native amino acids to produce gain-of-function p53 variants, is a super clever angle that could push this approach beyond simple restoration.

Overall, I really like this study, and the core idea and data are quite compelling and relevant. I just have a few additions and clarifications to elevate the manuscript further:

Response: We sincerely thank the reviewer for these positive comments and valuable suggestions.

1. The structural claims around deleterious substitutions like Y234Q and G266R are based on static models. I'd really like to see the authors quantify how those substitutions alter local flexibility or overall stability during the MD simulations. Just showing RMSD and per-residue RMSF across the DBD would make the modeling section far more convincing than visual inspection alone.

Response: We are grateful to the reviewer for this valuable suggestion. We analyzed the RMSD and RMSF results generated during the MD simulations. Interestingly, these previously identified deleterious variants did not show significant differences in these metrics compared with WT-p53 or neutral variants. This is likely due to the unique functional properties of p53: alterations in a few key amino acid residues that directly contact DNA can have catastrophic functional consequences, even if the overall structure of the p53 protein remains largely unchanged. For this reason, we focused our analysis on changes in the interaction between key amino acid residues and the DNA binding interface, an approach widely used in the field of p53 reactivating agents (PMID: 37018419).

2. Nowhere do the authors directly confirm that the intended amino acid is inserted at the site of readthrough. That's a big gap. A straightforward LC-MS/MS experiment on p53 pulled down from Calu-6 or Caco-2 cells could close this loop? It would directly validate the specificity of the sup-tRNA and rule out unintended wobble pairing or misincorporation.

Response: We are grateful to the reviewer for the valuable suggestion. The actual amino acids incorporated by the sup-tRNAs were verified by mass spectrometry (Supplementary Fig. 3, lines 134-139), which demonstrated that the sup-tRNAs used in this study enable the precise incorporation of specific amino acids at nonsense mutation sites, consistent with previous reports (PMID: 30778053, 39673265).

3. There's no check on sup-tRNA expression across constructs. That makes it hard to interpret read-through efficiency comparisons in Fig. 2b or Fig. 3a. It wouldn't take much (RT-qPCR or even Northern blot) for the authors to verify whether the differences they're seeing are biological or just a function of expression variability.

Response: We are grateful to the reviewer for this valuable suggestion. In this study, all sup-tRNAs were constructed using the identical vector backbone, promoter, and transcription unit architecture, and the molar amount of plasmid and total transfected DNA were kept consistent across all transfections to ensure

a nearly identical transcriptional initiation environment for each sup-tRNA. The nonsense readthrough efficiency of a sup-tRNA in cells is an integrated functional phenotype determined by both its intrinsic molecular properties and its actual intracellular expression level. These two factors are not independent of each other; rather, they are inextricably linked and together define the intrinsic readthrough capacity of the sup-tRNA. On one hand, different sup-tRNAs exhibit natural differences in their steady-state intracellular expression levels due to variations in their sequence, structure, processing and maturation efficiency, and stability. On the other hand, these differences in expression level are an inherent component of sup-tRNA function. Once delivered into cells, sup-tRNAs integrate into the endogenous cellular tRNA pool, and their transcription, processing, stability, and functional activity are dynamically regulated by the cellular translational machinery as a whole. Therefore, the directly measured readthrough efficiency in this study reflects the comprehensive functional performance of the suppressor tRNA in a bona fide cellular environment, which more closely mimics the physiological conditions of its potential future therapeutic application.

4. The computational selection framework (Fig. 2f) is presented as a black box. It would help to see even a small benchmark: say, how well the model predicted the best-performing sup-tRNA for 10 p53 nonsense variants, compared to what actually worked. Without that, it's hard to judge if the model meaningfully informed tRNA design.

Response: We thank the reviewer for pointing this out. We have updated the manuscript accordingly. We validated the robust performance of our computational selection framework for guiding the selection of effective sup-tRNAs through experimental assessments of p53 transcriptional activity and colony formation assays, the best-performing sup-tRNA actually worked well (**Supplementary Figs. 5-7**). Furthermore, to eliminate the "black box" perception of the model and improve its accessibility to the broader research community, we systematically applied this approach to 715 non-arginine/non-glutamine nonsense mutations across four core tumor suppressor genes (*TP53*, *RB1*, *PTEN*, and *APC*). For each mutation site, we performed *in silico* saturation mutagenesis to predict the functional impact of all possible missense variants generated by sup-tRNA-mediated readthrough. This comprehensive analysis yielded 122,265 pathogenicity predictions and gene-specific super-tRNA manuals, which provide: (i) Heatmaps visualizing the predicted pathogenicity scores for all amino acid substitutions at each nonsense mutation locus; (ii) Actionable recommendations for identifying optimal sup-tRNAs that convert stop codons into neutral or beneficial missense variants (**lines 257-269, Supplementary Information files: Super tRNA-TP53-Manual, Super tRNA-RB1-Manual, Super tRNA-PTEN-Manual, Super tRNA-APC-Manual**).

5. The *in vivo* work relies on intratumoral injection, which is perfectly fine for proof-of-concept. I'd love to see the authors test an LNP-based or EV-based delivery system *in vitro*, just something to show that sup-tRNAs can be delivered more broadly than with direct injection. That kind of experiment would make this feel closer to translational reality. But definitely not necessary.

Response: We are grateful to the reviewer for this valuable suggestion. We have updated the manuscript accordingly. We used lipid nanoparticles (LNPs) as the delivery vehicle for sup-tRNAs and validated their efficacy *in vivo* (**lines 445-479**). We have also provided a detailed description of the characterization of the LNP-tRNA formulations (**Supplementary Fig. 13, lines 445-456**), their production workflow (**lines 869-889**), and the administration dosage (**lines 899-905**). To further enhance the clinical relevance of this work, we evaluated the combination of sup-tRNA with the FDA-approved chemotherapeutic agent nab-paclitaxel, and observed robust therapeutic efficacy with this combination regimen (**Figure 6, lines 548-**

568).

This is a well-designed and exciting paper, and with a few additions like these, it could be a really definitive statement on the therapeutic use of engineered tRNAs! Overall, upon these minor changes, I will recommend acceptance.

Response: We sincerely thank the reviewer for the positive comments and valuable suggestions, which have greatly helped us improve the quality of this manuscript.

Reviewer #4 (Remarks to the Author):

“Restoration of tumor suppressor protein with enhanced activity using engineered tRNAs to induce tumor suppression”, by Kong et al.

The manuscript describes the generation of a series of sup-tRNAs for several p53 non-sense mutations and their effects on inducing p53 expression and the biological consequences thereof.

Overall, the concept of promoting readthrough at stop codons using various methods, including drugs like G418 has been previously shown for many genes. These further include the tumor suppressors p53 and PTEN. Moreover, the use of sup-tRNAs to overcome stop codons have also been well established (Ref 25 and 26 in this paper). As such, the conceptual novelty of the present findings is significantly reduced, as the authors have now tested a set of specific sup-tRNA for several p53 non-sense mutations to demonstrate readthrough.

The quality of the work needs significant improvement. Many controls are lacking – as an example, there is no demonstration that the sup-tRNAs are indeed in the tumor, or other organs, in the in vivo study. The global RNA seq analyses only used the cells with mutant p53, and do not include p53 null cells. This will be an important control to evaluate the effects (non-specific) of the sup-tRNAs. Moreover, demonstration that the sup-tRNAs that are generated for the indicated p53 mutations can or cannot work on other genes in the genome needs to be clarified (globally). This is crucial control to exclude non-specific effects, that are associated with G418.

Response: We thank the reviewer for these critical comments and valuable suggestions, which have guided our revisions to strengthen the manuscript. To directly address these concerns, we have performed additional dedicated in vivo validation experiments and comprehensive transcriptomic analyses. Specifically, in the p53-null tumor model, we found that sup-tRNA administration did not exert any detectable tumor suppressive effects, nor did it induce any treatment-related toxicity (**Supplementary Figs. 18-20, lines 488-513**). Furthermore, we performed global RNA seq analysis on tumor tissues from this p53-null model following sup-tRNA treatment, and the results confirmed that sup-tRNA exposure did not cause significant genome-wide dysregulation of other genes, nor did it induce non-specific perturbations of the p53 signaling pathway in the absence of mutant p53 (**Supplementary Fig. 19**). These data collectively demonstrate that the tumor suppressive effects of our sup-tRNA therapy are entirely dependent on the functional restoration of mutant p53, rather than off-target effects or non-specific toxicity.

The quality of the presentation of the data can be significantly improved, and in its current state, posed a challenge in the review process. Firstly, the fonts and figures are minute, even after enlarging on the screen. Secondly, very little details are given on the procedures. An example (among many) is that there is no information on how the tRNAs were delivered in vivo. There is a sentence in the M&M that the sup-

tRNAs were delivered by dendrimer nanoparticles. No reference or details are given.

The references to the figures in the text are not in order and some are missing. For e.g. after Fig 2, the authors refer to Fig 4, and then come back to Fig 3. More thought in the organisation of the manuscript is needed.

Response: We are grateful to the reviewer for the valuable suggestion. We have updated the manuscript accordingly. We have redrawn all figures and adjusted the font sizes to ensure clarity and readability. In the revised manuscript, we used lipid nanoparticles (LNPs) as the delivery vehicle for sup-tRNAs and validated their efficacy in vivo (lines 445-479). We have also provided a detailed description of the characterization of the LNP-tRNA formulations (Supplementary Fig. 13, lines 445-456), their production workflow (lines 869-889), and the administration dosage (lines 899-905). In addition, we have comprehensively reorganized the manuscript and renumbered the figures to ensure that all figures are cited in sequential order in the text.

Language can be significantly improved. An example (among many) is on the M&M on xenografts: “.....Caco-2 cells were injected into the substance of each mouse...” It is unclear what this means, and the M&M does not provide reliable information on how this is done. In the Intro section, the sentences on the driver ability are unclear to understand what the authors are trying to imply.

Response: We sincerely thank the reviewer for these valuable suggestions. We have redrawn nearly all of the result figures and enlisted the help of native English speakers to extensively revise the manuscript, significantly improving the quality of data presentation and overall readability. For the Materials and Methods section describing the xenograft models, we have revised the description to ensure clarity and comprehensibility (lines 890-914). For the section describing the driver ability of tumor suppressor genes, we thank the reviewer for pointing out the lack of clarity, and we have updated the manuscript accordingly. We have redrawn the relevant figures and provided a more detailed explanation of these results in the Results section (Figure 1a-b, lines 93-108).

Point-by-point response to the reviewers' comments

We are grateful to the editor and reviewers for their constructive and positive comments, which have greatly helped us improve the quality of the manuscript. We have carefully addressed all the issues raised. Below are our point-by-point responses to the reviewers' comments, with comments in black and responses in blue, respectively.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The revision has addressed many concerns from the previous review with additional experiments and discussion. Data presentation is also significantly improved. However, the current version still suffers from several issues.

Response:

We sincerely thank the reviewer for the positive comments and constructive suggestions. We have carefully reviewed all the remaining concerns and made targeted revisions throughout the manuscript. In addition, we have updated the manuscript title for improved clarity. We believe all raised issues have been fully addressed in the revised manuscript.

Major concerns:

1. The premise of using *in silico* predication to design specific suppressor tRNAs that introduce non-WT, activity-enhancing residues is a key claim, but it is not sufficiently supported by the data. Many experiments comparing suppressor tRNAs that introduce WT or non-WT residues only have p53 function data, but lack protein amount data showing how much p53 protein is restored (Fig. 4, Supplementary Fig. 5). P53 protein restoration should be quantified by western blot to see whether the amount of protein restoration, rather than the residue identity, is the major determinant for functional restoration.

Response: We are grateful to the reviewer for this valuable suggestion. In response, we have added quantitative Western blot analyses to assess p53 protein restoration levels (lines 191-198, lines 386-390, Supplementary Fig. 5e, Supplementary Fig. 11a). These data allowed us to evaluate the respective impacts of p53 protein abundance and residue identity on the functional recovery. The corresponding results and discussion have been revised accordingly (lines 191-198, lines 386-390). Taken together, these results support the hypothesis that the identity of the incorporated amino acid residue is a major determinant of p53 functional restoration.

2. The new data presented in Fig. 6 is based on a p53-null cell line with a p53-R210* transgene introduced by lentivirus transduction, but not cells with endogenous p53 nonsense mutations as in Fig. 5. Using the transgenic cell line poses a major caveat regarding how much p53 protein can be maximally restored, and whether it is relevant to the level of endogenous p53. For example, a strong promoter, multiple transgene copies, lack of mutant mRNA degradation by NMD can all artificially boost protein restoration and exaggerate efficacy. Therefore, the experiments in Fig. 6 should be repeated using the cell model(s) in Fig. 5.

Response: We thank the reviewer for this constructive comment. We agree that the original 4T1-*Trp53*^{R210*} syngeneic model has several limitations (e.g., exogenous promoter-driven expression, multi-copy integration and NMD evasion) because it relies on the lentiviral cDNA vector system. These features may artificially increase p53 restoration levels and consequently overestimate therapeutic efficacy (lines

590-598).

To address this concern, we performed additional *in vivo* validation using two cell-derived xenograft models (Calu-6 and Caco-2) bearing endogenous *TP53* nonsense mutations, with a dosing regimen consistent with that used in the 4T1-*Trp53*^{R210*} syngeneic model. We confirmed that sup-tRNAs effectively restore full-length p53 and suppress tumour growth in both models, and that combination with Nab-PTX produced the strongest antitumour effect (lines 598-622, Fig. 7a-g). Notably, p53 restoration levels in these endogenous models were lower than those observed in the cDNA transgenic system, while the core therapeutic conclusion remained robust (lines 623-630, Fig. 7h-k). We also observed a favorable safety profile based on body weight monitoring, histopathological examination of major organs and blood examination (lines 636-641, Supplementary Figs. 23-25).

All the new data have been integrated into the revised manuscript in Fig. 7 and Supplementary Figs. 23-25, and the corresponding findings are described in the Results section (lines 588-644). These additional experiments address the reviewer's insightful concern and strengthen the physiological relevance and translational value of our findings.

Minor suggestions:

1. Fig. 1e: why these tRNAs were selected from each family for testing? All glutamate tRNAs failed - please elaborate on this result.

Response: We thank the reviewer for this important question, which allows us to clarify both the rationale for tRNA selection and the mechanistic basis for the observed glutamate tRNA failure.

Part 1: Rationale for tRNA selection from each family

tRNAs tested in Fig. 1e were selected based on a two-step rationale:

Step 1: Selection of the amino acid families. We first analyzed the mutation spectra of key tumour suppressor genes (*TP53*, *APC*, *RB1*, *PTEN*) and found that that nonsense mutations in these tumour suppressor genes predominantly affect six amino acid families: arginine, glutamate, glutamine, tryptophan, tyrosine and serine. These six families collectively account for the majority of nonsense mutations in the tumor suppressor genes examined.

Step 2: Screening within each tRNA family. For each amino acid tRNA family, we tested available human tRNA isoacceptors/isodecoders from the genomic tRNA database (GtRNAdb).

During screening, we prioritized suppressor tRNAs requiring only a single base substitution to enable readthrough of premature termination codons corresponding to the respective amino acid. This design rationale was based on the hypothesis that minimal sequence modification of the tRNA anticodon loop region would cause minimal disruption to its native structure, translational fidelity and biochemical function. The complete screening data for all tested tRNAs are provided in Supplementary tables (Supporting Information-tRNA sequences and nomenclature). We also acknowledge this limitation in the manuscript. Although the sup-tRNAs employed in our study exhibit high fidelity and efficiency, further scaffold engineering can improve the suppression efficiency of certain sup-tRNAs (lines 690-694, PMID: 37258671).

Part 2: Elaboration on the failure of all glutamate tRNAs

Consistent with the reviewer's observation, all 12 human glutamate suppressor tRNAs tested exhibited uniformly low readthrough efficiencies (up to 1.37%), in stark contrast to other amino acid families

including arginine (up to 38.96%) and glutamine (up to 54.29%). This is a well-documented biological phenomenon with multiple mechanistic explanations:

1. The native glutamate tRNAs (such as tRNA^{Glu-1-1}_{CUC} and tRNA^{Glu-2-1}_{UUC}) translate codons beginning with G (GAG and GAA, respectively), whereas the anticodon-engineered sup-tRNAs are tasked with decoding a codon that begins with U (UAG). This replaces a strong G·C pair with a comparatively weaker U·A pair at the first position of the codon–anticodon interaction. Although nucleotides outside the anticodon triplet in the anticodon stem and loop (ASL) are evolutionarily tuned to balance codon–anticodon binding energies for native glutamate codons, these residues cannot offset the substantial affinity loss caused by the U·A base pair at the first codon position, resulting in inefficient UAG readthrough (PMID: 40539513, 27448410, 16828219).

2. Previous analyses have identified distinct consensus motifs for UNN (codon that begins with U)- and GNN (codon that begins with G)- decoding tRNAs that differed at several positions. At positions 32 and 38, nearly all UNN-decoding tRNAs possess a C32–A38 nucleotide pair. In contrast, nearly all GNN-decoding tRNAs possess U–C, C–U, U–U, or C–C pairs. These nucleotides at positions 32 and 38 are known to influence codon–anticodon affinity. This structural divergence implies that reengineering positions 32 and 38 to a C32–A38 combination would be required to restore sufficient binding affinity for the UAG codon (PMID: 40539513, 16828219, 16116437, 19305403).

3. The last three nucleotide pairs of the anticodon stem are important factors for stabilizing the stem's helical conformation (PMID:27448410). A strong consensus is observed only in the last two positions of the stem for both UNN- and GNN-decoding tRNAs. For UNN-decoding tRNAs, the base pair at position 31-39 was almost universally A31·U39 (85 out of 87 sequences). In contrast, GNN-decoding tRNAs predominantly harbored G31·C39 or C31·G39 pairs (110 out of 114 sequences) (PMID: 40539513).

Thus, single-base engineering in native glutamate tRNAs' anticodon loop is insufficient to generate glutamate suppressor tRNAs with high readthrough efficiency. This discrepancy is also a reason why all human glutamate suppressor tRNAs we tested displayed consistently low readthrough efficiency.

This limitation of glutamate suppressor tRNAs has been confirmed by Specht et al. (PMID: 40539513), who explicitly stated that glutamate (Glu) codons represent one of the most common sites for nonsense mutations, yet existing sup-tRNAs are ineffective at suppressing Glu-to-Stop mutations. Importantly, they addressed this limitation by rationally designing tRNA^{GluV13} with body sequence modifications beyond the anticodon (including optimization of the anticodon stem and loop), achieving greatly improved suppression efficiency.

Our study addresses this limitation through the super-tRNA strategy. As described in the manuscript, when the wild-type amino acid cannot be restored due to the low readthrough efficiency of the corresponding tRNA^{Glu_{UAG}}, high-efficiency alternative sup-tRNAs (such as tRNA^{Gln_{UAG}} and tRNA^{Ser_{UAG}}) can effectively restore p53 function, provided that the incorporated amino acid does not disrupt protein structure. This super-tRNA strategy effectively circumvents the inefficiency of glutamate sup-tRNAs by leveraging high-efficiency tRNAs from other amino acid families, thereby converting the nonsense mutation into a neutral variant.

2. New mass spectrometry data is shown, but lacks Methods information.

Response: We thank the reviewer for pointing this out. We have revised the mass spectrometry Methods accordingly to include detailed experimental procedures (**lines 802-833**).

3. Fig. 2f (and other figures): should "Being" be "Benign"?

Response: We sincerely thank the reviewer for raising this point. We agree with the reviewer's comment and have revised "Being" to "Benign" in the corresponding figures and manuscript (**Figure 2f, Supplementary Fig. 7a, lines 179, 944**).

4. Line 196: should those tRNAs targeting UAG?

Response: We sincerely thank the reviewer for pointing this out. We have carefully checked and corrected the description, and revised the relevant text to ensure accuracy throughout the manuscript (**lines 187-198**).

5. Line 256: should it be Supplementary Fig. 8b, not 9b?

Response: We sincerely thank the reviewer for pointing this out. We agree with the reviewer's comment and have carefully checked and corrected the reference in the manuscript (**line 255**).

6. Again, please make sure figures are called out in order. For example, Supplementary Fig. 18 and 19 now are mentioned before Supplementary Fig. 16 and 17.

Response: We are grateful to the reviewer for this valuable suggestion. We have carefully rearranged all relevant figures and revised the manuscript accordingly to ensure all figures are called out in the correct order.

Reviewer #2 (Remarks to the Author):

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

Response: We sincerely thank the reviewers for their valuable work and contributions to the peer review process.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to all of my comments. I recommend acceptance.

Response: We sincerely thank the reviewer for these positive comments and valuable suggestions, which have greatly helped us improve the quality of this manuscript.

Reviewer #5 - Replacement for Reviewer #4 (Remarks to the Author):

the authors have significantly improved the manuscript organisation, language and Figures. They have performed important additional experiments in accordance with referees criticism, which provide substantial evidence supporting author's conclusions.

Response: We are grateful to the reviewer for these positive comments and valuable contributions.

Point-by-point response to the reviewers' comments

We are grateful to the editor and reviewers for their constructive and positive comments, which have greatly helped us improve the quality of the manuscript. We have carefully addressed all the issues raised. Below are our point-by-point responses to the reviewers' comments, with comments in black and responses in blue, respectively.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all my concerns.

Response: We sincerely thank the reviewer for these positive comments and valuable suggestions.

Reviewer #2 (Remarks to the Author):

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

Response: We sincerely thank the reviewers for their valuable work and contributions to the peer review process.