nature portfolio

Peer Review File

Inhibition of tumor-intrinsic NAT10 enhances antitumor immunity by triggering type I interferon response via MYC/CDK2/DNMT1 pathway

Corresponding Author: Professor Daoxin Ma

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
This study investigates the role of NAT10, an RNA acetyltransferase, in regulating tumor immune response and explores strategies for antitumor immunotherapy through NAT10 repression combined with anti-PD1 therapy. The authors first demonstrate that high NAT10 expression correlates with worse prognosis in lung adenocarcinoma. Using two syngeneic models, murine lung tumor (TC1) and murine fibrosarcoma (MCA205), they show that repression of NAT10 activity by remodelin inhibits tumor growth in immunocompetent mice but not in immunocompromised mice. Increased immune cell infiltration, particularly CD8+ T cells, was observed in NAT10-downregulated human tumor samples and murine syngeneic tumors. The study also identifies a significant enrichment of the Type I IFN response in NAT10-depleted tumor cells. Mechanistically, the authors found that NAT10 promotes the acetylation and stability of several oncogene-encoded mRNAs, including MYC. They propose that downregulation of NAT10 regulates antitumor immunity through the MYC-CDK2-DNMT1 axis, enhancing dsRNA generation to induce an immune response via RIG-I signaling. The synergistic effect of NAT10 depletion and anti-PD1 therapy is demonstrated, and a therapeutic nanoparticle-encapsulated siRNA targeting NAT10 is developed. While the study presents intriguing findings, several major concerns temper the enthusiasm and some issues require clarification.
Major Issues:
1. The proposed NAT10-regulated immune response heavily relies on the concept that “DNMT1 inhibition induces dsRNA formation.” However, the study only cites a single reference (Oncoimmunology 2021;10:1989790), which does not directly show that DNMT1 inhibition induces dsRNA formation but only demonstrates a Type I IFN response. The manuscript lacks a detailed explanation or mechanism supporting how DNMT1 inhibition leads to dsRNA generation.
2. The study suggests that NAT10 regulates MYC RNA acetylation, influencing its stability and impacting antitumor immunity through a linear MYC-CDK2-DNMT1 signaling pathway. However, MYC is a well-known oncogene with numerous target genes beyond CDK2. The authors did not explore the impact of MYC RNA acetylation on other target genes, limiting the analysis to CDK2.
3. The immune response analysis moves quickly from RIG-I-mediated immune activation to a broad antitumor effect, including synergism with anti-PD1 and immune memory. A more detailed investigation of NAT10-mediated immune responses is needed.
Additional Points for Clarification:
1. The rationale for using a murine fibrosarcoma model should be clarified, specifically the relevance of NAT10 in human sarcomas or any particular biological characteristics of sarcomas related to NAT10 or dsRNA.
2. It is not convincing that downregulation of MYC and CDK2 primarily affects immune responses more than tumor growth, given their critical roles in cellular proliferation. The data also show inconsistencies; for instance, in Fig. S1A-B, the NAT10 inhibitor remodelin suppresses clonogenicity of murine tumor cell lines in vitro, yet remodelin only inhibits tumor growth in immunocompetent but not immunocompromised mice. If the tumor-suppressive effect of NAT10 inhibition is immune-dependent, why does in vitro clonogenicity differ? A similar inconsistency is observed in Fig. 2 and Fig. S2A, where NAT10 depletion reduces clonogenicity in vitro but affects tumor growth only in an immune-dependent manner.
3. The immune memory effect observed in Fig. 2I requires further clarification. The mechanism by which the sgNAT10 tumor vaccine induces immune memory should be thoroughly investigated.

4. The immunophenotyping in this study mostly relies on CD8 as a T cell marker in both flow cytometry and immunofluorescence, without further subtyping. Multicolor flow cytometry to categorize immune cells is essential, and quantification of multicolor immunofluorescence staining in Fig. 3D should be provided.
5. Fig. 5D: The rationale for focusing on MYC as the primary target of NAT10 regulation, without considering other potential factors, needs to be explained.
6. Given that NAT10 is currently the only known RNA ac4C writer, the study should address the broader impact of NAT10 on global RNA acetylation rather than limiting the discussion to one oncogene-encoded RNA.

Reviewer #2

(Remarks to the Author)
The manuscript by Liu et al. demonstrates that the inhibition of NAT10 enhances the antitumor immunity by increasing type I interferon responses. Liu and colleagues showed that tumor-intrinsic NAT10 loss reduces tumor growth in immune-competent mice but exerts a less pronounced effect in immune-deficient mice. Further, loss of NAT10 was associated with increased infiltration and activation of adaptive immune effectors. They identified Myc and type I interferon responses as downstream targets of NAT10 and provided evidence that inhibiting NAT10 with either small molecule inhibitor or PEI/PC7A/siNAT10 nanoparticles synergized with PD-1 blockade to enhance the anti-tumor immune response and repress tumor progression. Overall, this is an interesting study revealing the crucial role of tumor-intrinsic NAT10 in tumor immune microenvironment, which represents a promising target to enhance cancer immunotherapy. However, some specific concerns need to be addressed before the manuscript can be accepted for publication in Nature Communication.

Major comments:
1. It is unclear why the authors choose MCA205 and TC1 as their tumor models. What are the NAT expression levels in these two cell lines compare to other murine cancer cell lines?
2. In Figs. 1E - 1H, the authors showed some interesting results that low-dose Remodelin significantly inhibited tumor growth in immuno-competent B6 mice, but not in immuno-comprised nude mice. Does adoptive transfer of B6-derived T cells into nude mice restore the effects of Remodelin on tumor growth? Based on Fig. 1I, the difference in CD8 T cell infiltration between NAT-high and -low groups is pretty small although significant, and several other immune populations (e.g., B cells, pDCs, macrophages) also displayed significant or even more pronounced differences. The authors should provide a more comprehensive evaluation of different immune populations to screen out the major population mediating NAT10's effects. Alternatively, if adoptive T cell transfer restored Remodelin's effects in nude mice, it would also be a robust evidence that NAT10 depends on T cells to modulate anti-tumor immunity.
3. The authors showed in Fig. 2I that immunization of B6 mice with NAT10-deficient but not WT tumor cells led to later elimination of transplanted WT tumors growth. Does this effect also exist in nude mice? If so, does depletion of T cells in B6 mice abolish the preventive effects of NAT10-deficient tumor vaccine?
4. The data in Fig. 3E showed that whereas CD8+ T cell depletion markedly promoted NAT10-deficient tumor growth, there was still a significant difference between WT and NAT-deficient tumor size in mice depleted of CD8+ T cells. This indicates that NAT10 modulates the anti-tumor immune response not only depending on CD8+ T cells, but also other immune populations. The description in the text needs to be revised to more accurately interpret this result. Moreover, the authors showed later that NAT10 inhibition triggered type I interferon responses. The results would be more convincing it the authors could use IFNAR antibodies to prove it in vivo.
5. According to the GSEA results in Fig. 3A, 4A, and 4B, both type I (IFNα) and type II (IFNγ) IFN response pathways were significantly enriched in NAT10-deficient tumor cells. What is the reason to only focus on type 1 IFN pathway?
6. The authors proposed that NAT10 deficiency induces dsRNA formation via regulating DNMT1. However, a more detailed description of how DNMT1 affects dsRNA formation is warranted to better clarify this mechanism.
7. It has been reported that dsRNA can be recognized by the cytoplasmic RNA sensors RIG-I and MDA-5, thereby triggering the IFN-I response. Why did the authors silence RIG-I in sgNAT10 tumor cells instead of MDA-5?
8. Remodelin is a well-established NAT10 inhibitor known for its significant suppression of tumor cell proliferation. In addition, the author developed two novel delivery systems, SM102 and PEI/PC7A nanoparticles, to inhibit NAT10 in order to improve the efficacy, targeting precision, and safety of NAT10 inhibition. What are the pros and cons of Remodelin, PEI/PC7A, and SM102?
9. None of the experiments were performed on human cancer cells or using human-in-mouse xenotransplantation model. At the very least, some evidence from human cancer cell lines would greatly improve the clinical relevance of these findings.

Minor points:
1. More details are needed on the methods used to achieve Fig.1I .
2. Can overexpression of Myc and CDK2 in NAT10 deficient cells abrogate the tumor-intrinsic NAT10 deficiency mediated tumor immune rejection?
3. The authors should provide the sequences of guide oligonucleotides for the knockout target gene.
4. NAT10 deletion significantly inhibited the expression of CDK2 in tumor cells. The detection of CDK2 expression in tumor tissues would further strengthen the validity and robustness of the results.
5. What is the difference between SM102 and PEI/PC7A nanoparticles?
6. A ruler or a scale bar need to be included in the tumor images (Fig 7B, C).
7. The size distribution of nanoparticles protocol is unclear.

Reviewer #3

(Remarks to the Author)
In this manuscript, Liu et al. describe the role of NAT10-mediated N4-acetylcytidine modification in the activation of the MYC/CDK2/DNMT1 pathway, leading to the repression of the anti-tumor immune response. Post-transcriptional regulation of gene expression is increasingly recognized as a critical mechanism for immune system regulation, and this study potentially highlights the role of a specific post-transcriptional RNA modification in suppressing the immune response to lung adenocarcinoma. As such, it holds potential interest for audiences across several fields.
While the manuscript presents several novel and intriguing findings, it is notably hindered by a lack of transparent reporting of methods. The unavailability of primary data restricts the ability to accurately assess many of the claims. Furthermore, several key assertions made in the manuscript are not strongly supported by the data presented. In some cases, such as the claim regarding translational regulation of Myc mRNA, no supporting data is provided at all. In addition, the rational for some experimental design are not clear and there are some figures, which are not mentioned in the text. These shortcomings must be addressed with additional experimentation, access to primary data, and a more measured presentation of the conclusions.

- Information regarding the data presented in Figure 1C is scant. The authors mention “we collected lung cancer samples”. What sort of sample? What stages of tumor? How did the staining look like? This information, along with examples of the stained samples need to be presented, perhaps in supplementary data.

- Some of the claims of “significant” findings in the manuscript needs further verification, particularly with statistical analysis. For instance, the manuscript claims “that Remodelin significantly suppressed the proliferation of tumor cells (Figs. S1A, S1B).” I don’t dispute that Figures S1A & B show a reduction in clonogenicity, especially with 20 μM concentration, but to claim that these differences are “significant”, quantitation of the data and appropriate statistical analyses are needed.

- It is impossible to assess the quality of the RNA-Seq, acRIP-seq, and proteomics data presented in this manuscript without access to the original data or, at minimum, a tabulated summary of metrics such as read counts. The authors should deposit the original data in appropriate public repositories (e.g., GEO for RNA-Seq data and PRIDE for proteomics) and provide the processed data (e.g., DESeq results) as supplementary tables. These are standard practices that would allow both expert computational biologists and the general audience to fully evaluate and appreciate the findings of the study.

- There is no information regarding how the label-free quantitative proteomics was conducted, and it is unclear what criteria were used to consider a protein as differentially expressed in Fig. 5D. A similar issue exists for the RNA-Seq analyses. The authors should clarify what fold-change or statistical analyses, if any, were applied. Providing this information in full detail is crucial for a proper assessment of the results and ensuring future reproducibility.

- From the data presented in Figures 5A-D, it seems that acRIP-seq was performed in both WT and NAT10 knockout cells. However, neither the text nor the figure legend discusses the main differences in the assay outcomes between the two cell types. Given that NAT10 is the only known ac4C writer, did the authors observe a general absence of ac4C in the knockout cells? It is difficult to assess this with the provided information and the lack of original results or a complete list of enriched mRNAs.

- In lines 139-140, the authors suggest that “tumor-intrinsic NAT10 promotes tumor growth mainly mediated by inducing tumor immune evasion.” However, considering that their colony formation assays with NAT10-knockout cells (Figs. S2B-E) and with cells treated with Remodelin (Fig. S1A-B), as well as the analysis with nude mice (Fig. 2C-D) show a considerable and statistically significant reduction in clonogenicity and tumourigenecity of the NAT10-inhibited cells, NAT10 seem to have substantial immune-independent effect on these phenotypes and therefor the above statement should be toned down.

- What is the source of the anti-ac4C monoclonal antibody used in the acRIP-seq assay?

- The description of the dual luciferase assay for the analysis of the Myc 3´ UTR is unclear. The diagram in Fig. 5G shows the pEZX-MT06 vector, which contains both hLuc and R-Luc. However, the figure legend for Fig. 4H states, “Additionally, Renilla luciferase plasmids (30 ng/well) were co-transfected as a normalization control for transcription efficiency.” What is the rationale for this? The matter is further complicated by the fact that the Methods section for the dual luciferase assay (Line 666) describes the following: “The promoter activity of NAT10 in TC1 cells was assessed using a luciferase assay.” It is unclear why the NAT10 promoter activity is being tested here and how this was achieved using the pEZX-MT06 vector. Clarification on these points is needed.

- In line 251, the authors claim that “the results of acRIP-PCR also confirmed that NAT10 bound to the 3'UTR of MYC (Fig. 5I)”. It is not clear how this assay proves that NAT10 bound to the 3'UTR. There also seem to be a discrepancy between the text, which suggest this is an acRIP-PCR, and the legend, which states it as being a NAT10 RIP-qPCR.

- In lines 255-7, the authors claim that “Overall, these results demonstrate that NAT10 promotes the mRNA stability and translation efficiency of MYC via ac4C modification, and the ac4C peak in the 3’UTR region is responsible for mRNA stability”. What is the evidence for promotion of translation efficiency of Myc mRNA by NAT10? This claim needs supporting data, including the use of polysome profiling/RT-PCR.

- It is quite surprising that overexpression of either Myc or CDK2 fully restores the phenotypes associated with NAT10 knockout back to those of the parental cells (e.g., Fig. 6D). Does this mean that all oncogenic functions of NAT10 are solely due to the upregulation of MYC and the subsequent upregulation of CDK2 and that no other target mRNA of NAT10, aside from Myc, nor any downstream targets of MYC besides CDK2, contribute to the observed phenotype? This is a critical point to address, as it appears that other targets of NAT10 identified in this study (e.g., Fig. 5D) also affect the expression of key proinflammatory cytokines (e.g., GIGYF1; PMID: 39018414).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed most of the concerns raised by the reviewers. However, there is still room for improvement regarding the clinical relevance of the study.
1. For lung adenocarcinoma (LUAD), the prognostic impact of EGFR or other driver gene mutations and the use of tyrosine kinase inhibitors (TKIs) is substantial. The authors should take this into account when analyzing the LUAD clinical data.
2. The authors state that the use of a murine fibrosarcoma syngeneic cell line is intended to model "cold tumors." However, this approach has two key weaknesses: (1) there is no comparative analysis across multiple cancer types to support which tumors are "cold" or "hot," and (2) human sarcomas represent a highly heterogeneous group of diseases characterized by distinct fusion genes as drivers, which differ significantly from the syngeneic fibrosarcoma model used in this study. Incorporating analyses of human fibrosarcoma data and immune cell infiltration across multiple cancer types would improve the clinical relevance of the study.

Reviewer #2

(Remarks to the Author)
The author has addressed my concerns, and I have no further questions.

Reviewer #3

(Remarks to the Author)
The authors have made commendable efforts and have largely succeeded in addressing my comments on the first version of this manuscript. However, a few outstanding issues still need to be resolved

1- In response to my original comment “In line 251, the authors claim that “the results of acRIP-PCR also confirmed that NAT10 bound to the 3'UTR of MYC (Fig. 5I)”. It is not clear how this assay proves that NAT10 bound to the 3'UTR” the authors replied that “In Fig. 5I, an anti-NAT10 antibody-based RIP-PCR instead of acRIP-PCR (We are sorry for the mistake) experiment was conducted using primers specifically targeting the MYC 3’UTR, confirming the interaction between NAT10 and this region. We have updated the text with more explanation of how this assay proves that NAT10 bound to the 3'UTR (line 270).”
However, this response still does not address how the assay definitively proves binding to the 3´ UTR. The authors state that they used primers specific to the 3´ UTR, which only demonstrates that the pulled-down mRNA contains the 3´ UTR. It does not confirm that NAT10 binds specifically to the 3´ UTR. NAT10 could potentially bind to the CDS or 3´ UTR, and the primers would still amplify the 3´ UTR sequence.
In my opinion, the experimental design is flawed and cannot conclusively demonstrate binding to the 3´ UTR. To address this issue, the authors would need to use a control mRNA where the 3´ UTR is either mutated to disrupt the NAT10 binding site or entirely absent. If the hypothesis is correct, the RIP-PCR should only yield a positive result for the full-length cDNA and not for the mutant or truncated mRNA.

2- Regarding the claim of translational regulation of MYC mRNA by NAT10, the authors conducted a Ribo-Seq assay, which is appropriate and demonstrates reduced footprint levels on MYC mRNA. However, since the KO cells also exhibit lower MYC mRNA expression compared to the WT, the conclusion that the “translation efficiency” of MYC mRNA is reduced is not entirely accurate. I recommend the authors revise the manuscript to indicate that both mRNA expression and footprint levels are downregulated, leading to an overall decrease in protein expression and avoid the term “translation efficiency”, which would likely not be appropriate for the observed changes. Additionally, I would like to draw the authors' attention to the increased footprint levels in the 5′ UTR of MYC mRNA in KO cells. This observation could be related to activation of the ISR pathway, which, while beyond the scope of this manuscript, may be worth exploring in future studies.

3. In response to my last comment on the 1st version of the manuscript being overly focused on MYC and CDK2, the authors have expanded on their explanation regarding how restoring MYC or CDK2 expression rectified the observed phenotypes. While I do not question their observations or explanation, I believe it would be appropriate to acknowledge in the Discussion section that the MYC-CDK2 axis is likely only one of the downstream mechanisms through which NAT10 regulates the phenotype and that there are likely other potentially significant downstream factors that were not explored in detail.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed the raised concerns, and I am satisfied with their response and the revised manuscript.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns regarding the previous version of the manuscript, and I have no further questions.

Reviewer #1 (Remarks to the Author):

Comment 1:

This study investigates the role of NAT10, an RNA acetyltransferase, in regulating tumor immune response and explores strategies for antitumor immunotherapy through NAT10 repression combined with anti-PD1 therapy. The authors first demonstrate that high NAT10 expression correlates with worse prognosis in lung adenocarcinoma. Using two syngeneic models, murine lung tumor (TC1) and murine fibrosarcoma (MCA205), they show that repression of NAT10 activity by remodelin inhibits tumor growth in immunocompetent mice but not in immunocompromised mice. Increased immune cell infiltration, particularly CD8+ T cells, was observed in NAT10-downregulated human tumor samples and murine syngeneic tumors. The study also identifies a significant enrichment of the Type I IFN response in NAT10-depleted tumor cells. Mechanistically, the authors found that NAT10 promotes the acetylation and stability of several oncogene-encoded mRNAs, including MYC. They propose that downregulation of NAT10 regulates antitumor immunity through the MYC-CDK2-DNMT1 axis, enhancing dsRNA generation to induce an immune response via RIG-I signaling. The synergistic effect of NAT10 depletion and anti-PD1 therapy is demonstrated, and a therapeutic nanoparticle-encapsulated siRNA targeting NAT10 is developed. While the study presents intriguing findings, several major concerns temper the enthusiasm and some issues require clarification.

Response: We sincerely thank you for taking the time to provide such valuable and constructive feedback on our manuscript. Your insightful comments and suggestions have been incredibly helpful, leading us to make revisions that have greatly improved the rigor, clarity, and overall quality of our work. We deeply appreciate your professional guidance and thank you once again for your thoughtful and detailed review.

Comment 2:

Major Issues:

1. The proposed NAT10-regulated immune response heavily relies on the concept that "DNMT1 inhibition induces dsRNA formation." However, the study only cites a

single reference (Oncoimmunology 2021;10:1989790), which does not directly show that DNMT1 inhibition induces dsRNA formation but only demonstrates a Type I IFN response. The manuscript lacks a detailed explanation or mechanism supporting how DNMT1 inhibition leads to dsRNA generation.

Response: Thanks for your comments. As you said, we recognized that our initial explanation lacked sufficient depth. Therefore, we have addressed this concern by providing additional clarification and performed new experiments as follows:

(1) Literature Evidence:

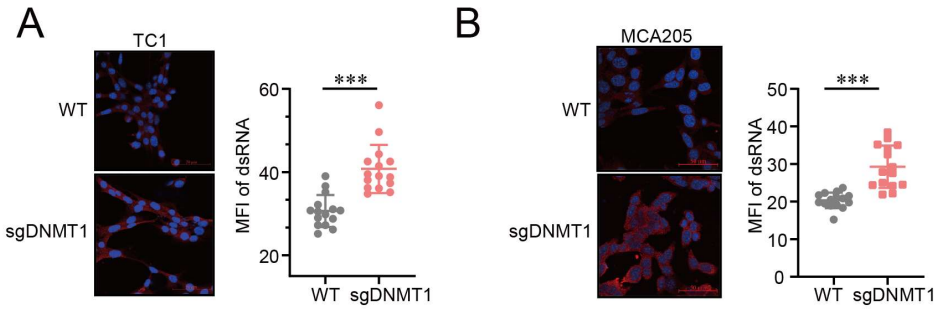
Study by KB Chiappinelli et.al (PMID: 26317466) suggests that DNA methyltransferase inhibitors (DNMTis), such as 5-azacytidine (Aza) and 5-aza-2'-deoxycytidine (Dac), can trigger an immune response in cancer cells (Response Figure 1). This response was mediated through the activation of cytosolic dsRNA sensors. Further investigations revealed DNMTis can induce the expression of ERV genes, which can form dsRNA and contribute to the interferon response . Additional literature also indicates that DNMT1 deficiency leads to hypomethylation, activation of ERVs, and subsequent production of dsRNA (PMID: 37991725). Now we have revised the manuscript and cited the references accordingly (lines 50-60, 342, 517-521).

[FIGURE REDACTED]

Response Figure 1. DNA methyltransferase inhibitors (DNMTis) trigger cytosolic sensing of double-stranded RNA (dsRNA) causing a type I interferon response and apoptosis. Chiappinelli, Katherine B. et al. Cell, Volume 162, Issue 5, 974 - 986, PMID: 26317466.

(2) Experimental Evidence:

As DNMT1 inhibition has been reported to trigger type I interferon (IFN-I) responses by inducing the formation of double-stranded RNA (dsRNA), we quantified cytoplasmic dsRNA levels using the dsRNA-specific J2 antibody (Response Figure 2). The results below demonstrated a significantly higher abundance of dsRNA in DNMT1 knockout cells compared to WT cells, indicating DNMT1 inhibition leads to dsRNA generation.



Response Figure 2: DNMT1 knockout leads to higher abundance of cytoplasmic dsRNA in TC1 cell (A) and MCA205 (B).

Comment 3:

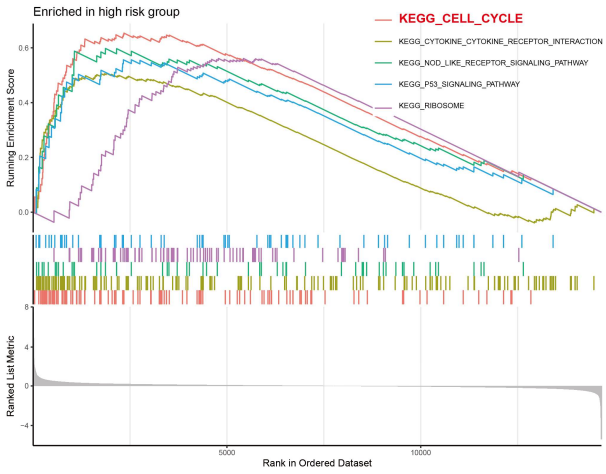
2. The study suggests that NAT10 regulates MYC RNA acetylation, influencing its stability and impacting antitumor immunity through a linear MYC-CDK2-DNMT1 signaling pathway. However, MYC is a well-known oncogene with numerous target genes beyond CDK2. The authors did not explore the impact of MYC RNA acetylation on other target genes, limiting the analysis to CDK2.

Response: Thank you for your valuable feedback. As you pointed out, MYC is a well-known oncogene with a broad range of target genes beyond CDK2. We acknowledge that our manuscript overly focused on CDK2 as a downstream target of MYC. To address this, we have made the following clarifications and revisions:

(1) CDK2 as a Downstream Target of NAT10 and MYC:

We first analyzed RNA-seq data from sgNAT10 and sgMYC cell lines and identified CDK2 as a common downregulated gene (Fig. S6A), consistent with findings from previous studies (PMID: 33541412). Western blot analysis further confirmed reduced CDK2 expression in both sgNAT10 and sgMYC cell lines (Figs. 5E, 6C and S6C). Similar to MYC (Response Figure 3), CDK2 plays a critical role in

regulating cell cycle progression and modulating tumor immune responses (Figs. S5 and S6E, F).



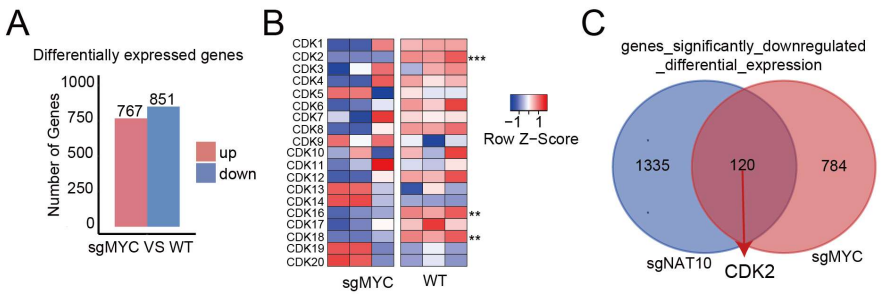
Response Figure 3: Our analysis of the LUAD cohort in the TCGA database revealed that patients with high Myc expression are predominantly enriched in the cell cycle signaling pathway.

These results suggest that CDK2 serves as a critical downstream target of MYC, which is why our study mainly focus on CDK2.

(2) Other Downstream Targets of MYC:

As you mentioned, MYC is a well-known oncogene with numerous target genes beyond CDK2. To investigate this broader regulatory network, we newly performed RNA-seq analysis on sgMyc TC1 cells. This newly conducted analysis revealed significant transcriptional changes, with 767 genes upregulated and 851 genes downregulated, underscoring the diverse regulatory roles of MYC (Response Figure 4A). In addition, we analyzed RNA-seq data from sgNAT10 and sgMYC cell lines and identified CDK2 as a commonly downregulated gene, along with other candidates such as Psd4, Nsg1, Smpd3, Psph, Edn1, and Batf3 and so on (Response Figure 4C). These genes may have important roles in the MYC regulatory network, which require further investigation.

We appreciate your valuable comment again. With your helpful suggestion, we included the detailed discussion of these findings is included in the revised manuscript (lines 305-311, 500-506).



Response Figure 4: (A) Differential Gene Expression Profiles Following Myc Deletion. (B) Altered CDK Family Gene Expression in Response to Myc Deletion. (C) Shared Downregulated Genes Following Myc and NAT10 Deletion.

Comment 4:

3. The immune response analysis moves quickly from RIG-I-mediated immune activation to a broad antitumor effect, including synergism with anti-PD1 and immune memory. A more detailed investigation of NAT10-mediated immune responses is needed.

Response: Thank you for your valuable suggestion. Our RNA-seq analysis of NAT10-deficient tumor cells revealed significant upregulation of type I IFN signaling pathways, primarily driven by RIG-I recognition of dsRNA (Figs. S4A, B; S6K, L). Type I IFNs are known to enhance dendritic cell (DC) antigen presentation and activate adaptive immunity (PMID: 34800368). Consistent with this, we observed increased DC (Fig. S3F) and $CD8^+$ T cell (Figs. S3A, B) populations in NAT10-deficient tumors, while no differences on NK cells, macrophages, Treg cells, neutrophils, monocytes, Th1 cells and Th17 cells (Figs. S3G-L), suggesting enhanced antigen presentation and cytotoxic T cell activation. Given the critical role of $CD8^+$ T cells and checkpoint blockade in immunotherapy, we tested the combination of NAT10 inhibition and anti-PD1 therapy. In vivo experiments showed that this combination significantly suppressed tumor growth compared to either treatment administered individually (Fig. 7A, B). These findings indicate that NAT10 depletion enhances immune responses, from innate activation to adaptive immunity, and synergizes with checkpoint blockade, potentially inducing durable immune memory.

We appreciate your helpful comment again. With your suggestion, to make the transition from RIG-I-mediated immune activation to a broad antitumor effect

smoother, we revised the manuscript to clarify more detailed investigation of NAT10-mediated immune responses (lines 359-361, Figs. S3F-L)

Comment 5:

Additional Points for Clarification:

1. The rationale for using a murine fibrosarcoma model should be clarified, specifically the relevance of NAT10 in human sarcomas or any particular biological characteristics of sarcomas related to NAT10 or dsRNA.

Response: We appreciate your insightful comment. The purpose of our study is to investigate the effects and mechanisms of NAT10 inhibition in antitumor immunity. Therefore, we chose the cancers with the following characteristics: a "cold" tumor type with low immune cell infiltration, high tumorigenic potential, and poor therapeutic response. We initially selected lung cancer for our study, as it meets the relevant characteristics and is the most prevalent cancer type. Building on our findings in lung cancer, we extended our investigation to assess the relevance of NAT10 expression in other cancer types. Fibrosarcoma, which shares these characteristics, was chosen as a suitable model for studying NAT10-mediated antitumor immunity. Moreover, our experiments showed that inhibition of NAT10 could lead to the increase of dsRNA production in fibrosarcoma cells (Fig 6E), further supporting the relevance of NAT10 and dsRNA in sarcoma. These findings support the hypothesis that NAT10 plays a critical role in regulating immune activation across diverse cancer types. Given the limited availability of human sarcoma samples, our findings were primarily validated in lung cancer patients. We have addressed this limitation in the revised manuscript (lines 566-569, page 20).

Comment 6:

2. It is not convincing that downregulation of MYC and CDK2 primarily affects immune responses more than tumor growth, given their critical roles in cellular proliferation. The data also show inconsistencies; for instance, in Fig. S1A-B, the NAT10 inhibitor remodelin suppresses clonogenicity of murine tumor cell lines in vitro, yet remodelin only inhibits tumor growth in immunocompetent but not immunocompromised mice. If the tumor-suppressive effect of NAT10 inhibition is immune-dependent, why does in vitro clonogenicity differ? A similar inconsistency is

observed in Fig. 2 and Fig. S2A, where NAT10 depletion reduces clonogenicity in vitro but affects tumor growth only in an immune-dependent manner.

Response: Thank you for your careful, rigorous and valuable comments. As you said, in our manuscript, it appeared that downregulation of MYC and CDK2 primarily affects immune responses more than tumor growth，and that NAT10 depletion reduces clonogenicity in vitro but affects tumor growth only in an immune-dependent manner. We appreciate your deep insight and feel very sorry for our lack of detailed clarification in our previous manuscript. We address your concerns as follows:

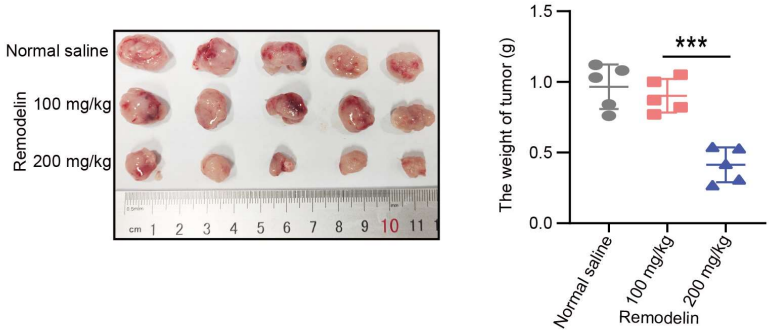
(1) Regarding MYC and CDK2 and their effects on immune responses vs. tumor growth:

As you mentioned, MYC and CDK2 play critical roles in cellular proliferation. Moreover, both MYC and CDK2 are also involved in modulating immune responses (PMID: 36323660; PMID: 39469865). In our study, we aimed to investigate how the deficiency of MYC or CDK2 suppresses tumor growth through the host immune response. Therefore, our animal experiments were primarily focused on assessing the immune response. Based on your suggestion, we have revised the manuscript to clarify this (lines 257-259, 289-291).

(2) Addressing inconsistencies between *in vitro* and *in vivo* effects of NAT10 inhibition:

In vitro, we used higher concentrations of remodelin, which significantly suppressed clonogenicity in murine tumor cell lines (Figs. S1B-C). Conversely, *in vivo* experiments were conducted using low-dose remodelin to primarily investigate its immunomodulatory effects, which resulted in limited cytotoxicity. This explains why remodelin effectively inhibited tumor growth in immunocompetent mice via immune activation (Figs. 1E-F), but showed no significant effect in immunocompromised mice (Figs. 1G-H). Your comment is quite important for the improvement of our manuscript. Therefore, to further address your concern, we performed new experiments to administer low-dose (oral gavage for the first 5 days at a dose of 100 mg/kg) and high-dose (200 mg/kg) remodelin to immunocompromised mice (Nude/Nude) and observed that high-dose remodelin significantly inhibited tumor growth (Response Figure 5), suggesting in vivo cytotoxic effects at higher concentrations. This dose-dependent observation aligns with findings in other studies,

such as those showing low-dose HDAC inhibitors activating immune responses in immunocompetent mice but lacking efficacy in immunodeficient models (PMID: 36898011). Based on your suggestion, we have revised the manuscript to clarify this (lines 460-472).



Response Figure 5：High-dose (200 mg/kg) remodelin significantly inhibited tumor growth compared to low-dose (100 mg/kg) and Normal saline in Nude/Nude mice.

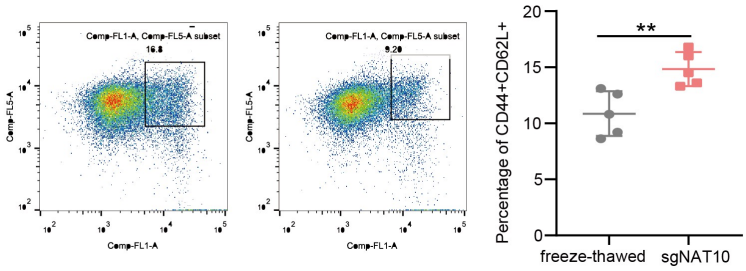
Comment 7:

3. The immune memory effect observed in Fig. 2I requires further clarification. The mechanism by which the sgNAT10 tumor vaccine induces immune memory should be thoroughly investigated.

Response: Thank you for your valuable comment. We apologize for the oversimplified description of the immune memory effect in Fig. 2I. This figure explores the role of sgNAT10 in inducing anti-cancer immune memory and its potential as a future tumor vaccine for cancer treatment.

To further address your concern, we performed new experiments by analyzing memory T cells in immunized C57BL/6N mice. The results by flow cytometry of inguinal lymph nodes revealed a significant increase in memory T cells in mice immunized with live sgNAT10 tumor cells compared to those treated with freeze-thawed WT tumor cells ,as shown in the Response Figure 6. Moreover, we also investigated whether T cell depletion in C57BL/6N mice could impair the preventive effects of NAT10-deficient tumor vaccines. We subcutaneously immunized C57BL/6N mice with either live sgNAT10 tumor cells or freeze-thawed WT tumor cells on the left side. In immunized mice, live WT tumor cells were inoculated, and T

cell function was blocked using an anti-CD8 antibody. The results demonstrated that $CD8^+$ T cell depletion completely abolished the preventive effects of the NAT10-deficient tumor vaccine, as tumors developed on the left flank (Figs. S2G, S2H). These additional experiments and descriptions have been included in the revised manuscript (lines 163-168).



Response Figure 6：C57BL/6N mice were subcutaneously immunized with either live sgNAT10 tumor cells or freeze-thawed WT tumor cells. Fourteen days post-immunization, the proportion of memory T cells in the inguinal lymph nodes was assessed by flow cytometry.

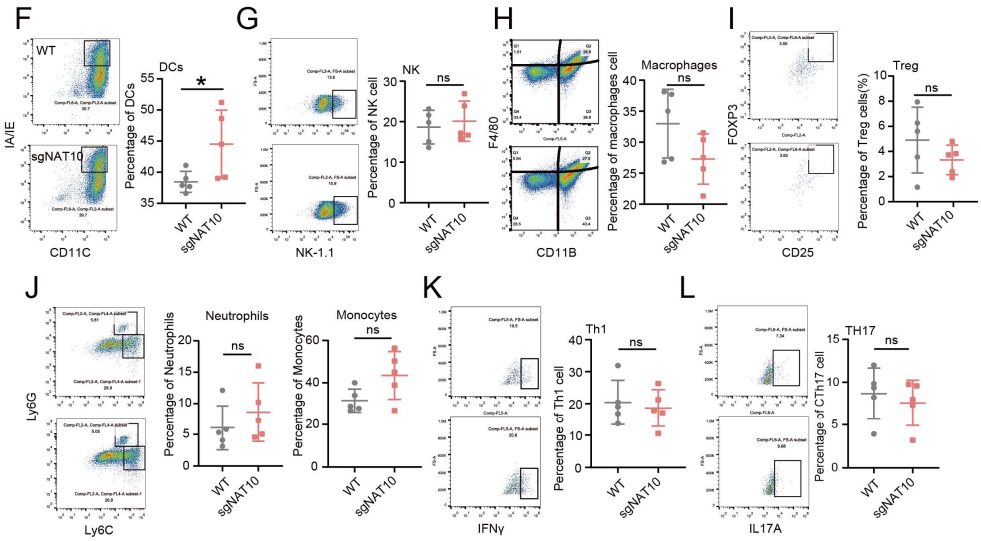
Comment 8:

4. The immunophenotyping in this study mostly relies on CD8 as a T cell marker in both flow cytometry and immunofluorescence, without further subtyping. Multicolor flow cytometry to categorize immune cells is essential, and quantification of multicolor immunofluorescence staining in Fig. 3D should be provided.

Response: Thank you for your valuable comments. Based on your helpful suggestion, we conducted new experiments by establishing mice tumor model followed by using multicolor flow cytometry to categorize immune cells, including DCs, NK cells, neutrophils, monocytes, Th1, Th17, and Treg cells. Our analysis revealed a significant elevation of DCs in sgNAT10 tumor tissues compared to WT tissues. This increase may result from NAT10 loss in tumor cells, which likely enhances interferon secretion and chemokine production, promoting DC recruitment. Other immune cell types did not exhibit statistically significant difference (Response Figure 7, Updated Figs. S3F-L). These findings and additional experiments have been incorporated into the revised manuscript (lines 208–212, 756-761; Figs. S3F–L).

Additionally, according to your valuable suggestion, we quantified multicolor immunofluorescence staining (Fig. 3D). Among the immune cells analyzed, CD8+ T cells exhibited the most significant differences between groups. We included this

quantification in the updated Fig. 3D.



Response Figure 7：Immune cell profiling via flow cytometry. Mice were were sacrificed at appropriate time, and the tumors were collected and separated into single cells. Briefly, tumors were excised and scissor minced. After filtering with strainer, the cell suspensions were stimulated with brefeldin A (BFA, PeproTech, 10 mg/mL), phorbol myristate acetate (PMA, PeproTech, 100 μg/mL) and ionomycin (PeproTech, 1mg/mL) at 37°C for 4 h. For the cell surface staining, cells were stained for cell markers including CD45, CD8a, CD11c/IAIE (F), NK1.1 (G), F4/80 (H), Foxp3 (I), LY6C/G (J), IFN-γ (K), and IL-17A (L). Cells were was analyzed using a Gallios flow cytometer (Beckman Coulter, USA) and the results were analyzed by Flowjo. Statistical significance was evaluated using an unpaired Student's t-test, with *p < 0.05 indicating significance.

Comment 9:

5. Fig. 5D: The rationale for focusing on MYC as the primary target of NAT10 regulation, without considering other potential factors, needs to be explained.

Response: Thank you for your insightful comment. We apologize for the oversimplified explanation in the original manuscript.

(1) Identification of MYC as a candidate target:

To investigate NAT10 regulation, we conducted acRIP-seq and proteomics analyses in NAT10-deficient cells, identifying 125 genes with reduced mRNA acetylation and 323 with decreased protein expression. Seven candidate genes (Phf2, Myc, Wwc2, Kmt2a, Gigyf1, Timeless, and Nufip2) showed reductions in both mRNA acetylation and protein levels.

(2) Selection of MYC as the primary target:

Our findings demonstrate that NAT10 regulates not only cell proliferation but also the tumor immune response. Among the candidate genes, MYC was prioritized due to its well-documented dual role in promoting cell proliferation and modulating antitumor immunity (PMID: 34508258). These characteristics, supported by extensive literature (PMID: 35962189), provide strong evidence for MYC as a critical target of NAT10 regulation.

Moreover, with your helpful suggestion, we included this explanation in the revised manuscript to clarify the rationale for selecting MYC as the primary target (lines 257-259, 495-500).

Comment 10:

6. Given that NAT10 is currently the only known RNA ac4C writer, the study should address the broader impact of NAT10 on global RNA acetylation rather than limiting the discussion to one oncogene-encoded RNA.

Response: Thank you for your insightful comment. As our study primarily focused on the mechanism by which NAT10 regulates the tumor immune microenvironment, we specifically investigated key downstream genes that influence immune responses. Through bioinformatics analysis and various experimental approaches, we identified Myc as a potential critical target gene downstream of NAT10. While our research emphasizes Myc's role in immune modulation, we fully acknowledge the broader impact of NAT10 on global RNA acetylation. As you suggested, we have included in the discussion section that future investigations will explore these broader effects, including global RNA acetylation patterns, to provide a more comprehensive understanding of NAT10's cellular functions (lines 257-259, 500-506).

Reviewer #2 (Remarks to the Author):

The manuscript by Liu et al. demonstrates that the inhibition of NAT10 enhances the antitumor immunity by increasing type I interferon responses. Liu and colleagues showed that tumor-intrinsic NAT10 loss reduces tumor growth in immune-competent mice but exerts a less pronounced effect in immune-deficient mice. Further, loss of NAT10 was associated with increased infiltration and activation of adaptive immune

effectors. They identified Myc and type I interferon responses as downstream targets of NAT10 and provided evidence that inhibiting NAT10 with either small molecule inhibitor or PEI/PC7A/siNAT10 nanoparticles synergized with PD-1 blockade to enhance the anti-tumor immune response and repress tumor progression. Overall, this is an interesting study revealing the crucial role of tumor-intrinsic NAT10 in tumor immune microenvironment, which represents a promising target to enhance cancer immunotherapy. However, some specific concerns need to be addressed before the manuscript can be accepted for publication in Nature Communication.

Response: We sincerely thank you for your thorough evaluation of our manuscript and for recognizing the potential significance of our findings. We greatly appreciate your insightful comments and constructive feedback, which will be invaluable in refining and strengthening our work. Below, we address the specific concerns you raised:

Major comments:

1. It is unclear why the authors choose MCA205 and TC1 as their tumor models. What are the NAT expression levels in these two cell lines compare to other murine cancer cell lines?

Response: Thank you for your insightful question. Lung cancer, a highly prevalent and lethal malignancy, often evades immune surveillance through mechanisms like PD-L1 expression, making it an ideal model for studying immune dysregulation and improving immunotherapy. We focused on NAT10 modulating immune responses in the tumor microenvironment. We found that NAT10 could modulates immune responses in the tumor microenvironment, influencing immunotherapy effectiveness. We investigated whether NAT10's role in reshaping the tumor immune microenvironment extends to other tumor types. We selected fibrosarcoma, a "cold" tumor with characteristics similar to lung cancer, and found that NAT10 also regulates immune responses in this tumor type, further supporting its broad role in immune modulation. The TC1 (lung cancer) and MCA205 (fibrosarcoma) cell lines are characterized by high tumorigenicity and low immunogenicity, making them ideal models for investigating the immune response in the context of host-tumor interactions. We have added this additional explanation and experiments in the revised manuscript (lines 560-569).

2. In Figs. 1E - 1H, the authors showed some interesting results that low-dose Remodelin significantly inhibited tumor growth in immuno-competent B6 mice, but not in immuno-comprised nude mice. Does adoptive transfer of B6-derived T cells into nude mice restore the effects of Remodelin on tumor growth? Based on Fig. 1I, the difference in CD8 T cell infiltration between NAT-high and -low groups is pretty small although significant, and several other immune populations (e.g., B cells, pDCs, macrophages) also displayed significant or even more pronounced differences. The authors should provide a more comprehensive evaluation of different immune populations to screen out the major population mediating NAT10's effects. Alternatively, if adoptive T cell transfer restored Remodelin's effects in nude mice, it would also be a robust evidence that NAT10 depends on T cells to modulate anti-tumor immunity.

Response: Thank you for your insightful comments. We appreciate your suggestion to further explore the role of T cells and other immune populations in mediating the effects of Remodelin. We address your concerns as follows:

(1) Adoptive Transfer of T cells in Nude Mice:

As shown in Figs. 1E-1H, our results indicate that low-dose Remodelin significantly inhibits tumor growth in immuno-competent B6 mice, but not in immuno-compromised nude mice (Nude/nude, which lack mature T lymphocytes).

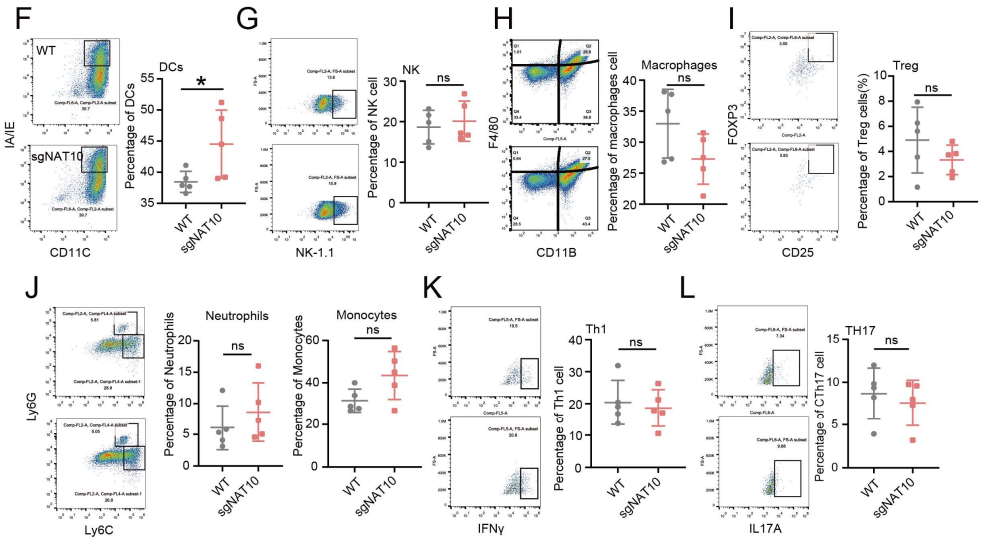
Mechanistic studies suggested that the anti-tumor effects of NAT10 deficiency are mediated by type I interferon secretion, which could enhances dendritic cell (DC) antigen presentation and activates $CD8^+$ T cells.

Adoptively transferring B6-derived T cells into nude mice to assess whether this can restore the impact of Remodelin on tumor growth is indeed an excellent approach to demonstrate the critical role of T cells. However, the Nude/nude mice we used are on Balb/c background, and we are uncertain whether C57BL/6N-derived T cells can function effectively in Nude mice. Nevertheless, to validate the pivotal role of T cells, we performed various experiments, including flow cytometry, immunofluorescence, and $CD8^+$ T cell-depletion experiments (Fig 3E).

These findings underscore the pivotal role of a functional adaptive immune system, particularly $CD8^+$ T cells, in driving the anti-tumor effects of Remodelin.

(2) Regarding immune cell profiling via flow cytometry:

We performed additional analyses using flow cytometry to profile various immune cell populations, including dendritic cells (DCs), natural killer (NK) cells, neutrophils, monocytes, Th1, Th17, and Treg cells. Notably, DC cell infiltration was elevated in sgNAT10 tumor tissues compared to WT tissues (Response Figure 7, Updated Figs. S3F-L). This elevation is potentially due to the loss of NAT10 in tumor cells, which may enhance interferon secretion and stimulate chemokine production, thereby promoting DC cell recruitment. Although some immune cell populations exhibited increased infiltration in the sgNAT10 group compared to the WT group, the differences were not statistically significant. We have added this additional explanation and experiments in the revised manuscript (lines 208–212, 756-761; Figs. S3F–L).



Response Figure 7：Immune cell profiling via flow cytometry. Mice were were sacrificed at appropriate time, and the tumors were collected and separated into single cells. Briefly, tumors were excised and scissor minced. After filtering with strainer, the cell suspensions were stimulated with brefeldin A (BFA, PeproTech, 10 mg/mL), phorbol myristate acetate (PMA, PeproTech, 100 μg/mL) and ionomycin (PeproTech, 1mg/mL) at 37°C for 4 h. For the cell surface staining, cells were stained for cell markers including CD45, CD8a, CD11c/IAIE (F), NK1.1 (G), F4/80 (H), Foxp3 (I), LY6C/G (J), IFN-γ (K), and IL-17A (L). Cells were was analyzed using a Gallios flow cytometer (Beckman Coulter, USA) and the results were analyzed by Flowjo. Statistical significance was evaluated using an unpaired Student's t-test, with *p < 0.05 indicating significance.

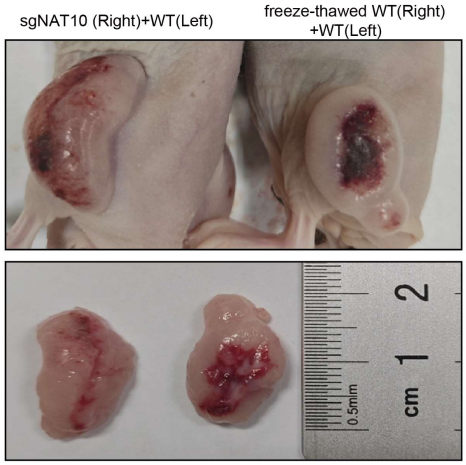
3. The authors showed in Fig. 2I that immunization of B6 mice with NAT10-deficient but not WT tumor cells led to later elimination of transplanted WT tumors growth.

Does this effect also exist in nude mice? If so, does depletion of T cells in B6 mice abolish the preventive effects of NAT10-deficient tumor vaccine?

Response: Thank you for your thoughtful questions and for highlighting an important aspect of our study. Below, We address the specific concerns regarding the effects observed in Fig. 2I and the role of T cells in mediating the preventive effects of NAT10-deficient tumor vaccines:

(1) Effect in Nude Mice:

We performed a similar experiment in nude mice, where we immunized them with either NAT10-deficient or freeze-thawed WT tumor cells. Interestingly, unlike in B6 mice, nude mice did not exhibit the same preventive effect against WT tumor growth following immunization with NAT10-deficient tumor cells (Response Figure 8). This suggests that the immune response, particularly T cells, plays a crucial role in the observed protective effect in immunocompetent mice.

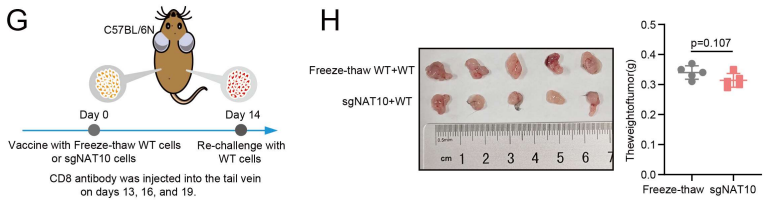


Response Figure 8：C57BL/6N mice were subcutaneously immunized with either live sgNAT10 tumor cells or freeze-thawed WT tumor cells on the left side, followed by re-challenging with comparable numbers of live WT tumor cells on the right side after one week.

(2) T Cell Depletion in B6 Mice:

In response to your second question, we tested whether T cell depletion in B6 mice could abolish the preventive effects of NAT10-deficient tumor vaccines. We conducted the experiment as follows: we divided the mice into two groups, one group receiving NAT10-deficient tumor cells and the other receiving freeze-thawed WT tumor cells. After 14 days, both groups were challenged with WT tumor cells on the

left flank. Notably, we depleted T cells in the B6 mice receiving NAT10-deficient tumor cells using CD8 antibodies (Response Figure 9, Updated Figs. S2G, S2H). The results showed that $CD8^+$ T cell depletion in the B6 mice completely abolished the preventive effects of the NAT10-deficient tumor vaccine, as tumors grew on the left flank of both groups.



Response Figure 9：(G and H) C57BL/6N mice were subcutaneously immunized at the left flank with equal numbers of sgNAT10 tumor cells or freeze-thawed WT tumor cells (control). Freezing and thawing were performed three times. Fourteen days after immunization, an equivalent number of live WT tumor cells were subcutaneously transplanted into the right flank of immunized mice. CD8 antibody was injected into the tail vein on days 13, 16, and 19. n=5 tumors per each group.

These findings provide robust evidence that T cells are essential for the observed preventive effects of NAT10-deficient tumor cells and suggest that the immune system, particularly $CD8^+$ T cells, is crucial in mediating the anti-tumor immunity induced by NAT10 loss. We included these additional results in the revised manuscript (lines 163-168, Figs. S2G, S2H).

4. The data in Fig. 3E showed that whereas CD8+ T cell depletion markedly promoted NAT10-deficient tumor growth, there was still a significant difference between WT and NAT-deficient tumor size in mice depleted of CD8+ T cells. This indicates that NAT10 modulates the anti-tumor immune response not only depending on CD8+ T cells, but also other immune populations. The description in the text needs to be revised to more accurately interpret this result. Moreover, the authors showed later that NAT10 inhibition triggered type I interferon responses. The results would be more convincing if the authors could use IFNAR antibodies to prove it in vivo.

Response: Thank you for your valuable feedback and insightful comments. We have carefully considered your points and made the following revisions to the manuscript:

(1) Interpretation of Results in Fig. 3E:

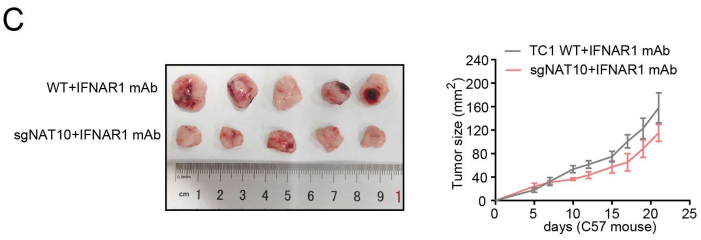
We acknowledge the observation that, even in $CD8^+$ T cell-depleted mice, there remains a significant difference in tumor size between WT and NAT10-deficient tumors. This finding indicates that the loss of NAT10 not only impacts the immune microenvironment and $CD8^+$ T cell activity but also directly influences tumor cell proliferation, as observed in the nude mice (Fig 2C, D). The observed significant difference in tumor size between WT and NAT10-deficient tumors in in mice depleted of CD8+ T cells can be attributed to the intrinsic proliferative capabilities of NAT10. To address this, we have revised the description in the manuscript to more accurately convey that NAT10 deficiency suppresses tumor cell proliferation, which contributes to the observed differences in tumor size (lines 195-197).

(2) Additional Immune Cell Involvement:

We agree that immune populations beyond $CD8^+$ T cells may contribute to the anti-tumor immune response in NAT10-deficient tumors. In response to this suggestion, we have included additional data analyzing the infiltration of other immune cell populations, including dendritic cells (DCs), natural killer (NK) cells, neutrophils, monocytes, Th1, Th17, and Treg cells (Response Figure 1, Updated Figures S3F-L). Notably, DC cell infiltration was elevated in sgNAT10 tumor tissues compared to WT tissues. This elevation is potentially due to the loss of NAT10 in tumor cells, which may enhance interferon secretion and stimulate chemokine production, thereby promoting DC cell recruitment. Although some immune cell populations exhibited increased infiltration in the sgNAT10 group compared to the WT group, the differences were not statistically significant. We have added this additional explanation and experiments in the revised manuscript (lines 208–212, 756-761; Figs. S3F–L).

(3) Use of IFNAR Antibodies to Validate Type I Interferon Responses:

We fully agree with your suggestion that the results would be more convincing if we could demonstrate the involvement of type I interferon signaling in vivo. To strengthen our findings, we conducted additional experiments using IFNAR antibodies to block type I interferon signaling in vivo. As shown in Response Figure 10 (Updated Fig. S4C), the results confirm that blocking the type I interferon pathway abolished the tumor-suppressive effects of NAT10 deficiency, further supporting the critical role of type I interferon responses in mediating the anti-tumor effects of NAT10 inhibition. We include these additional data in the revised manuscript (line 235, Fig. S4C).



Response Figure 10：(C) Tumor growth curves and weight of WT or sgNAT10 TC1 cells inoculated into C57BL/6N mice (n=5) treated with neutralizing antibodies targeting IFNAR1 or PBS injected every 2–3 days.

5. According to the GSEA results in Fig. 3A, 4A, and 4B, both type I (IFNα) and type II (IFNγ) IFN response pathways were significantly enriched in NAT10-deficient tumor cells. What is the reason to only focus on type 1 IFN pathway?

Response: Thank you for your thoughtful question regarding the focus on the type I IFN pathway in our study. We provide the following clarification based on the differences in experimental design and analytical methods:

(1) Regarding Fig. 3A:

The data presented in Fig. 3A were derived from tumor tissues, where immune cell activation predominantly drives the type II (IFNγ) IFN response. This pathway is critical for facilitating cytotoxic activity against tumor cells within the immune microenvironment. As a result, the enrichment of IFNγ signaling reflects the immune-mediated effects observed in the tumor context.

(2) Regarding Fig. 4A and 4B:

These figures are based on RNA-seq analyses of cultured tumor cells, which exclude contributions from the immune microenvironment. GSEA results showed enrichment of both type I and type II IFN response pathways. However, further GO enrichment analysis revealed a specific and significant association with the type I signaling pathway (Fig S4A,B). This suggests that in the absence of immune cells, the observed response is more strongly driven by intrinsic cellular mechanisms related to type I IFN signaling.

(3) Use of IFNAR Antibodies to Validate Type I Interferon Responses:

We fully agree with your suggestion that the results would be more convincing if we could demonstrate the involvement of type I interferon signaling in vivo. To

strengthen our findings, we conducted additional experiments using IFNAR antibodies to block type I interferon signaling in vivo. As shown in Response Figure 4 (Updated Fig. S4C), the results confirm that blocking the type I interferon pathway abolished the tumor-suppressive effects of NAT10 deficiency, further supporting the critical role of type I interferon responses in mediating the anti-tumor effects of NAT10 inhibition.

We include the data in the revised manuscript (line 218, Figs. 4A, 4B and S4C). We hope this explanation clarifies our focus on the type I interferon pathway in tumor cell, and we appreciate your suggestion to consider the involvement of type II IFN in future investigations.

6. The authors proposed that NAT10 deficiency induces dsRNA formation via regulating DNMT1. However, a more detailed description of how DNMT1 affects dsRNA formation is warranted to better clarify this mechanism.

Response: As you said, we recognized that our initial explanation lacked sufficient depth. Therefore, we have addressed this concern by providing additional clarification and performed new experiments as follows:

(1) Literature Evidence:

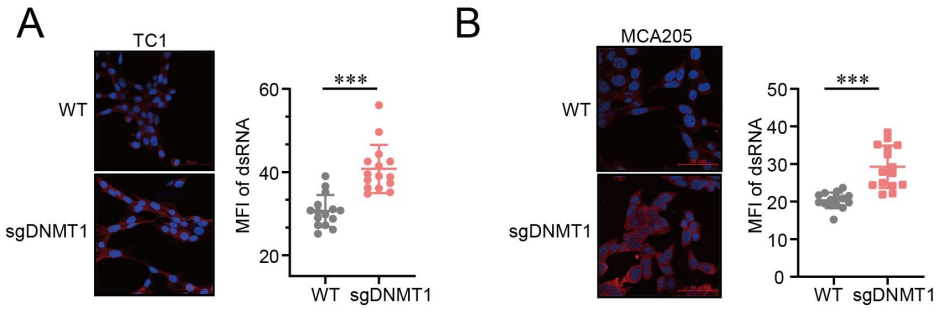
Study by KB Chiappinelli et.al (PMID: 26317466) suggests that DNA methyltransferase inhibitors (DNMTis), such as 5-azacytidine (Aza) and 5-aza-2'-deoxycytidine (Dac), can trigger an immune response in cancer cells (Response Figure 11). This response was mediated through the activation of cytosolic dsRNA sensors. Further investigations revealed DNMTis can induce the expression of ERV genes, which can form dsRNA and contribute to the interferon response . Additional literature also indicates that DNMT1 deficiency leads to hypomethylation, activation of ERVs, and subsequent production of dsRNA (PMID: 37991725). Now we have revised the manuscript and cited the references accordingly (lines 50-60, 342, 517-521).

[FIGURE REDACTED]

Response Figure 11. DNA methyltransferase inhibitors (DNMTis) trigger cytosolic sensing of double-stranded RNA (dsRNA) causing a type I interferon response and apoptosis. Chiappinelli, Katherine B. et al. Cell, Volume 162, Issue 5, 974 - 986, PMID: 26317466.

(2) Experimental Evidence:

As DNMT1 inhibition has been reported to trigger type I interferon (IFN-I) responses by inducing the formation of double-stranded RNA (dsRNA), we quantified cytoplasmic dsRNA levels using the dsRNA-specific J2 antibody (Response Figure 12). The results below demonstrated a significantly higher abundance of dsRNA in DNMT1 knockout cells compared to WT cells.



Response Figure 12: DNMT1 knockout leads to higher abundance of cytoplasmic dsRNA in TC1 cell (A) and MCA205 (B).

7. It has been reported that dsRNA can be recognized by the cytoplasmic RNA sensors RIG-I and MDA-5, thereby triggering the IFN-I response. Why did the authors silence RIG-I in sgNAT10 tumor cells instead of MDA-5?

Response: Thank you for your excellent question. We chose to focus on RIG-I rather

than MDA-5 for the following reasons:

Our preliminary data indicated that RIG-I, rather than MDA-5, showed a more pronounced association with the altered signaling pathway in NAT10-deficient cells (Figs S6K, L). Additionally, RIG-I is known to be a key receptor in recognizing cytoplasmic RNA, and our analysis suggested it might play a more significant role in mediating the IFN-I response in this context.

To further validate this, we performed additional experiments in which we knocked out the RIG-I receptor in NAT10-deficient cell lines, and we observed a significant reduction in the type I interferon signaling pathway, confirming that RIG-I is crucial for mediating the observed immune response (Fig 6F).

However, we recognize the importance of MDA-5 in dsRNA recognition and plan to explore its role in future experiments to provide a more comprehensive understanding of the IFN-I response in NAT10-deficient tumors. We update the manuscript to reflect this rationale for choosing RIG-I (lines 348-354, Fig. 6).

8. Remodelin is a well-established NAT10 inhibitor known for its significant suppression of tumor cell proliferation. In addition, the author developed two novel delivery systems, SM102 and PEI/PC7A nanoparticles, to inhibit NAT10 in order to improve the efficacy, targeting precision, and safety of NAT10 inhibition. What are the pros and cons of Remodelin, PEI/PC7A, and SM102?

Response: Thank you for your thoughtful question. Below is a summary of the pros and cons of Remodelin, PEI/PC7A nanoparticles, and SM102 in the context of NAT10 inhibition:

Remodelin:

Pros: (1) Well-established as a NAT10 inhibitor with a proven ability to suppress tumor cell proliferation. (2) Easy to administer, as it is typically delivered orally.

Cons: (1) Low absorption efficiency, which limits its inhibitory effect in vivo. (2) Suboptimal for achieving precise targeting and sufficient efficacy in animal models.

PEI/PC7A Nanoparticles:

Pros: (1) A technically mature delivery system with excellent performance in enhancing drug delivery efficacy. (2) Provides improved targeting precision and ensures effective NAT10 inhibition at the tumor site.

Cons: (1) Requires more complex preparation compared to Remodelin. (2) Potential

challenges in large-scale production and clinical translation.

SM102:

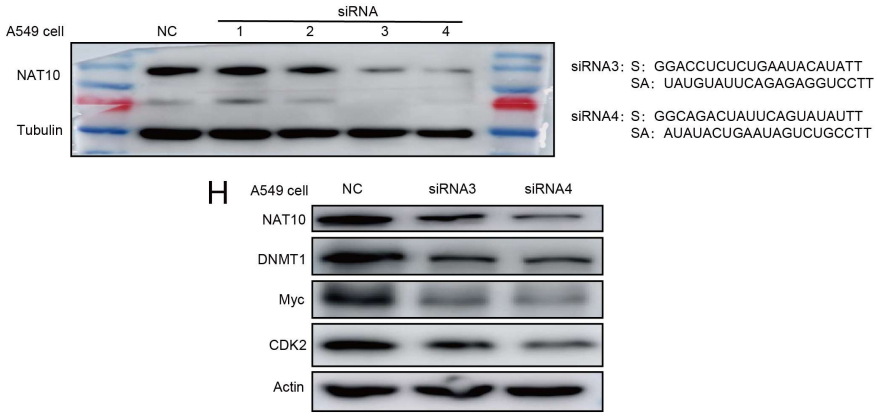
Pros: (1) Demonstrates high delivery efficiency with advanced targeting precision. (2) Offers a safer alternative with minimal off-target effects.

Cons: (1) Still relatively novel and requires additional validation in preclinical and clinical settings. (2) Like PEI/PC7A, the preparation process is more complex than for Remodelin.

In summary, while Remodelin is convenient and well-studied, its efficacy is limited in vivo due to poor absorption. PEI/PC7A and SM102 represent more advanced delivery systems that overcome these limitations, offering higher efficacy and precision. However, they involve more intricate preparation processes and require further validation for widespread application. We update the manuscript to reflect this rationale for choosing two novel delivery systems (lines 394-402).

9. None of the experiments were performed on human cancer cells or using human-in-mouse xenotransplantation model. At the very least, some evidence from human cancer cell lines would greatly improve the clinical relevance of these findings.

Response: Thank you for your insightful comment. We appreciate your suggestion to include evidence from human cancer cell lines to enhance the clinical relevance of our findings. According to your helpful suggestion, we have performed new experiments by using the human lung cancer cell line A549. We transfected A549 with siNAT10 to downregulate NAT10, then determine the expression of downstream proteins. Our results showed that inhibiting NAT10 can decrease the levels of downstream proteins, Myc, CDK2 and DNMT1 (Response Figure 13, Updated Fig. S6H). We included these results in the new version (lines 330-332, Fig. S6H).



Response Figure 13: (H) Western blot analysis of NAT10, DNMT1, MYC and CDK2 in matched WT and siNAT10 A549 tumor cells. β-actin was used as a loading control.

Minor points:

1. More details are needed on the methods used to achieve Fig.1I.

Response: Thank you for pointing this out. We have added more detailed descriptions of the methods used to generate Fig. 1I in the Materials and Methods section of the revised manuscript (lines 578-588). We hope this additional information clarifies the experimental procedures.

2. Can overexpression of Myc and CDK2 in NAT10 deficient cells abrogate the tumor-intrinsic NAT10 deficiency mediated tumor immune rejection?

Response: Thank you for your question. In our study, we overexpressed Myc and CDK2 in NAT10-deficient tumor cells and transplanted them subcutaneously into mice. We observed that the overexpression of Myc and CDK2 enabled these cells to form tumors (Figs. S5E-F, S6G), suggesting that Myc and CDK2 can partially abrogate the tumor-intrinsic NAT10 deficiency-mediated tumor suppression.

3. The authors should provide the sequences of guide oligonucleotides for the knockout target gene.

Response: Thank you for your suggestion. We have included the sequences of the guide oligonucleotides used for the knockout of the target gene in supplementary

materials of the revised manuscript (Table S1).

Table S1. Sequence of Guide RNA (sgRNA) oligonucleotides.

Gene	Forward	Reverse
Nat10	CACCGCAGCTGTACACGATGACTA	AAACTAGTCATCGTGTACAGCTGC
Myc	CACCGGACGTAGCGACCGCAACAT	AAACATGTTGCGGTCGCTACGTCC
Cdk2	CACCGAGGCCACTCACGTGTCGAGC	AAACGCTCGACACGTGAGTGGCCTC
Rig-I	CACCGATGTCTTATCAGATCCGACA	AAACTGTCGGATCTGATAAGACATC

4. NAT10 deletion significantly inhibited the expression of CDK2 in tumor cells. The detection of CDK2 expression in tumor tissues would further strengthen the validity and robustness of the results.

Response: Thank you for your insightful suggestion. To address this, we conducted additional analyses to strengthen the validity and robustness of our findings regarding the relationship between NAT10 and CDK2 expression.

(1) RNA-seq Analysis obtained from Tumor Tissues:

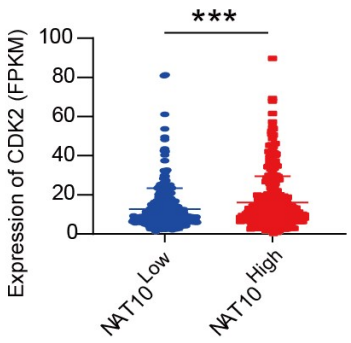
Our RNA-seq data analysis from tumor tissues revealed that NAT10 deletion significantly reduced the expression of CDK2 at the transcript level, as indicated by a marked decrease in CDK2 counts. This finding supports the regulatory role of NAT10 in CDK2 expression (line 180, Fig 3C).

(2) TCGA Database Analysis:

To further validate this observation, we analyzed data from the TCGA database. Lung cancer patients were stratified into high and low NAT10 expression groups. Consistently, we found that patients in the low NAT10 expression group exhibited significantly reduced CDK2 expression compared to those in the high NAT10 expression group (Response Figure 14).

These complementary approaches reinforce the link between NAT10 and CDK2 expression, highlighting the potential of NAT10 as a regulator of CDK2 in tumor cells. We have included these additional findings in the revised manuscript and supplementary materials to provide a more comprehensive evaluation.

Thank you again for your valuable feedback, which helped us strengthen our study.



Response Figure 14: The expression levels of CDK2 were assessed in relation to high and low NAT10 expression groups, using data from TCGA.

5. What is the difference between SM102 and PEI/PC7A nanoparticles?

Response: Thanks for your comments, SM102 is a lipid used in the formulation of lipid nanoparticles for RNA delivery, particularly in mRNA vaccines, while PEI/PC7A is a cationic polymer commonly used for DNA and RNA delivery. They both serve the purpose of delivering nucleic acids into cells but operate through different mechanisms, with SM102 being part of a lipid nanoparticle system and PEI/PC7A being a polymer that forms complexes with nucleic acids. We updated the manuscript to reflect the differences between the two novel delivery systems (lines 392-396).

6. A ruler or a scale bar need to be included in the tumor images (Fig 7B, C).

Response: Thank you for your feedback. We had included a ruler in the tumor images (Figs. 7B, C) to ensure proper measurement and clarity. The revised figure is updated accordingly in the new version of the manuscript.

7. The size distribution of nanoparticles protocol is unclear.

Response: Thank you for highlighting this point, and we apologize for any confusion caused. To clarify the nanoparticle size distribution protocol, the key steps are as follows:

a) Nanoparticles are dispersed in PBS to ensure a stable and uniform suspension.

b) Dynamic Light Scattering (DLS) is employed to measure the intensity-weighted size distribution. The size distribution is calculated using algorithms such as CONTIN or cumulant analysis.

c) The results are presented as a histogram, illustrating the proportion of particles within different size ranges. The polydispersity index (PDI) serves as a measure of the size distribution's breadth; a lower PDI (approaching 0) indicates a narrow and uniform distribution, while a higher PDI reflects greater variability in particle size.

In the context of drug development, accurate characterization of nanoparticle size is critical for assessing the stability and uniformity of drug carriers, which are pivotal to ensuring the effectiveness and safety of drug delivery systems.

We included the size distribution of nanoparticles protocol in the new version (lines 915-922). Thank you once again for your valuable feedback, and we look forward to your further evaluation.

Reviewer #3 (Remarks to the Author):

Comment 1:

In this manuscript, Liu et al. describe the role of NAT10-mediated N4-acetylcytidine modification in the activation of the MYC/CDK2/DNMT1 pathway, leading to the repression of the anti-tumor immune response. Post-transcriptional regulation of gene expression is increasingly recognized as a critical mechanism for immune system regulation, and this study potentially highlights the role of a specific post-transcriptional RNA modification in suppressing the immune response to lung adenocarcinoma. As such, it holds potential interest for audiences across several fields. While the manuscript presents several novel and intriguing findings, it is notably hindered by a lack of transparent reporting of methods. The unavailability of primary data restricts the ability to accurately assess many of the claims. Furthermore, several key assertions made in the manuscript are not strongly supported by the data presented. In some cases, such as the claim regarding translational regulation of Myc mRNA, no supporting data is provided at all. In addition, the rational for some experimental design are not clear and there are some figures, which are not mentioned in the text. These shortcomings must be addressed with additional experimentation, access to primary data, and a more measured presentation of the conclusions.

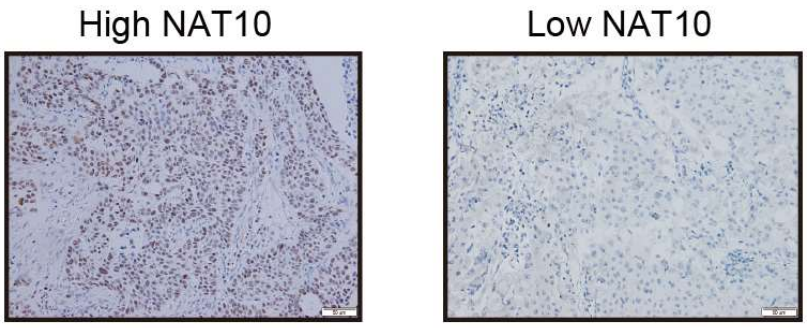
Response: We sincerely thank you for your comprehensive evaluation of our manuscript and for acknowledging the potential significance of our findings in elucidating the role of NAT10-mediated N4-acetylcytidine modification in immune regulation. Your constructive feedback has been invaluable in helping us identify key areas for improvement.

Comment 2:

- Information regarding the data presented in Figure 1C is scant. The authors mention "we collected lung cancer samples". What sort of sample? What stages of tumor? How did the staining look like? This information, along with examples of the stained samples need to be presented, perhaps in supplementary data.

Response: Thank you very much for your valuable comments. Regarding the sample information, the lung cancer samples we collected were formalin-fixed, paraffin-embedded (FFPE) tumor tissues, including adenocarcinoma and squamous cell carcinoma. These tumors represented a range of stages from I to IV, ensuring a comprehensive disease spectrum. As suggested, we have included detailed information of the patient samples, including age, sex, smoking history, sample type, and tumor stage in the supplementary data 1.

We are sorry for the lack of detailed information on the staining in Fig. 1C. Immunohistochemical staining was conducted to assess NAT10 expression in these samples, with positive staining predominantly localized in the nucleus (Response Figure 15). In response to your suggestion, we have added a more detailed description of the staining and included representative images in the revised manuscript (lines 591-600, Fig. S1A).



Response Figure 15: Immunohistochemical analysis of NAT10 expression in patient-derived lung cancer samples.

Comment 3:

- Some of the claims of "significant" findings in the manuscript needs further verification, particularly with statistical analysis. For instance, the manuscript claims "that Remodelin significantly suppressed the proliferation of tumor cells (Figs. S1A, S1B)." I don't dispute that Figures S1A & B show a reduction in clonogenicity, especially with 20 μM concentration, but to claim that these differences are "significant", quantitation of the data and appropriate statistical analyses are needed.

Response: Thanks a lot for your comment. With your valuable suggestion, we checked through the manuscript and verified the "significant" findings with statistical analysis. As for the old Figs. S1A, S1B, we quantified and statistically analyzed the data from three independent replicate experiments, and found the differences to be statistically significant. We included these results in the new Figure. S1B and S1C.

Comment 4:

- It is impossible to assess the quality of the RNA-Seq, acRIP-seq, and proteomics data presented in this manuscript without access to the original data or, at minimum, a tabulated summary of metrics such as read counts. The authors should deposit the original data in appropriate public repositories (e.g., GEO for RNA-Seq data and PRIDE for proteomics) and provide the processed data (e.g., DESeq results) as supplementary tables. These are standard practices that would allow both expert computational biologists and the general audience to fully evaluate and appreciate the findings of the study.

Response: Thank you for your valuable feedback. Based on your suggestions, we have made the following revisions to the manuscript:

(1) Deposition of Original Data:

We have deposited the original datasets in publicly accessible repositories to ensure transparency and reproducibility. Specifically: RNA-seq, acRIP-seq and Ribo-seq datasets have been deposited in the GEO database (GSE285518 and GSE275283). Proteomics data have been deposited in the PRIDE database (PXD059463).

(2) Processed Data:

We have provided detailed processed data as supplementary data, which include tables from RNA-seq, acRIP-seq, Ribo-seq, and 4D Label-free Proteomics. These supplementary tables now feature tabulated summaries, such as read counts and differentially expressed gene results, to facilitate assessment by both expert computational biologists and the general audience. Furthermore, we have updated the "Materials and Methods" section to include a comprehensive description of the data analysis procedures used for high-throughput sequencing (lines 666-680, 850-870; supplementary data 2-8).

Comment 5:

- There is no information regarding how the label-free quantitative proteomics was conducted, and it is unclear what criteria were used to consider a protein as differentially expressed in Fig. 5D. A similar issue exists for the RNA-Seq analyses. The authors should clarify what fold-change or statistical analyses, if any, were applied. Providing this information in full detail is crucial for a proper assessment of the results and ensuring future reproducibility.

Response: We sincerely appreciate your valuable comments. Based on your helpful suggestions, we have included detailed information on the methodology for label-free quantitative proteomics, along with the criteria used to identify differentially expressed proteins in the new manuscript (lines 666-680).

Moreover, with your helpful suggestion, we provided details regarding the RNA-seq analyses, including fold-change thresholds and statistical methods in the new version (lines 690-700, page 23).

Comment 6:

- From the data presented in Figures 5A-D, it seems that acRIP-seq was performed in both WT and NAT10 knockout cells. However, neither the text nor the figure legend discusses the main differences in the assay outcomes between the two cell types. Given that NAT10 is the only known ac4C writer, did the authors observe a general absence of ac4C in the knockout cells? It is difficult to assess this with the provided information and the lack of original results or a complete list of enriched mRNAs.

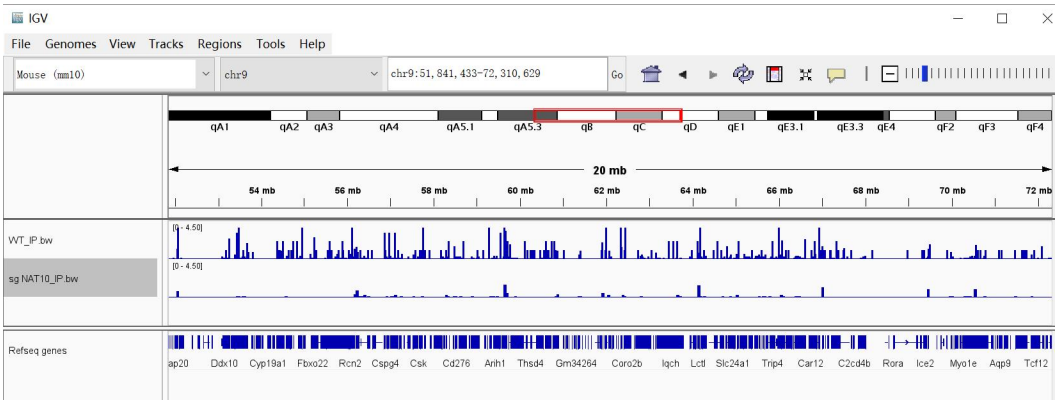
Response: Thank you very much for your thoughtful and detailed comments.

(1) acRIP-seq Performed in WT and NAT10 Knockout Cells:

We acknowledge that the rationale for using both WT and NAT10 knockout cells in our acRIP-seq experiments was not sufficiently clarified. The ac4C antibody (ab252215) used in our study, sourced from Abcam, is widely utilized to detect mRNA acetylation. NAT10 is currently the only known mRNA acetyltransferase, and existing literatures (PMID: 37349316; PMID: 37816771) suggest that genes identified through pull-down assays with this antibody are preliminarily regarded as targets of NAT10-mediated acetylation. To explore this further, we conducted acRIP-seq in both WT cells (with normal NAT10 expression) and NAT10 knockout cells, aiming to provide an initial identification of mRNAs potentially acetylated by NAT10.

(2) Differences Between WT and NAT10 Knockout Cells:

In our acRIP-seq experiments, we observed distinct differences between WT and sgNAT10 cells. Using IGV software, we found that the acetylated mRNAs enriched in WT cells were significantly much more abundant compared to those in sgNAT10 cells (Response Figure 16), consistent with the established role of NAT10 as a major contributor to ac4C deposition. We included this information in the revised manuscript to ensure clarity (line 245).



Response Figure 16: The acRIP-seq data for WT and sgNAT10 cell lines were visualized using the IGV software.

(3) Original Data and Supplementary Information:

Thank you for emphasizing the importance of providing more comprehensive data. In response, we have uploaded the original sequencing data to the GEO database (GSE285518) and included a detailed table of enriched mRNAs as a supplementary file (lines 243-245, supplementary data 5).

Comment 7:

- In lines 139-140, the authors suggest that "tumor-intrinsic NAT10 promotes tumor growth mainly mediated by inducing tumor immune evasion." However, considering that their colony formation assays with NAT10-knockout cells (Figs. S2B-E) and with cells treated with Remodelin (Fig. S1A-B), as well as the analysis with nude mice (Fig. 2C-D) show a considerable and statistically significant reduction in clonogenicity and tumourigenecity of the NAT10-inhibited cells, NAT10 seem to have substantial immune-independent effect on these phenotypes and therefor the above statement should be toned down.

Response: Thank you for your careful and valuable comment. With your helpful suggestion, we toned down the statement to "tumor-intrinsic NAT10 promotes tumor growth **partly** mediated by inducing tumor immune evasion." (line 111) .

Comment 8:

- What is the source of the anti-ac4C monoclonal antibody used in the acRIP-seq assay?

Response: In our study, we utilized the Anti- ac4C antibody from Abcam (catalog number: ab252215). We added this information in the revised manuscript to ensure clarity and transparency (line 831).

Comment 9a:

- The description of the dual luciferase assay for the analysis of the Myc 3′ UTR is unclear. The diagram in Fig. 5G shows the pEZX-MT06 vector, which contains both hLuc and R-Luc. However, the figure legend for Fig. 4H states, "Additionally, Renilla luciferase plasmids (30 ng/well) were co-transfected as a normalization control for transcription efficiency." What is the rationale for this?

Response: Thank you very much for your careful and rigorous comments. The dual-luciferase assay is a widely used biochemical technique designed to measure the activity of two different luciferase enzymes-commonly firefly luciferase (Photinus pyralis) and Renilla luciferase (Renilla reniformis)-in a single sample. In our study, we constructed the wild-type and mutant Myc gene sequences into the pEZX-MT06 reporter plasmids. The pEZX-MT06 reporter plasmid includes both luciferase and Renilla luciferase. However, when we used the dual-luciferase reporter system to measure luciferase and Renilla luciferase activity, we observed strong luciferase activity, but slightly weaker Renilla luciferase activity, resulting in lower accuracy. In order to be more rigorous and accurate, we additionally used Renilla luciferase plasmids as a reference for transfection efficiency between groups during the experiment.

Comment 9b:

The matter is further complicated by the fact that the Methods section for the dual luciferase assay (Line 666) describes the following: "The promoter activity of NAT10 in TC1 cells was assessed using a luciferase assay." It is unclear why the NAT10 promoter activity is being tested here and how this was achieved using the pEZX-MT06 vector. Clarification on these points is needed.

Response: Thanks a lot for your careful comment. We are very sorry for the descriptive error. The sentence "The promoter activity of NAT10 in TC1 cells was

assessed using a luciferase assay" is imprecise. The purpose of this experiment was to investigate the mRNA acetylation modification sites mediated by NAT10 in TC1 cells. We have corrected this erroneous description (line 811).

Comment 10a:

- In line 251, the authors claim that “the results of acRIP-PCR also confirmed that NAT10 bound to the 3'UTR of MYC (Fig. 5I)”. It is not clear how this assay proves that NAT10 bound to the 3'UTR.

Response: Thank you for your meticulous observation. In Fig. 5I, an anti-NAT10 antibody-based RIP-PCR instead of acRIP-PCR (We are sorry for the mistake) experiment was conducted using primers specifically targeting the MYC 3’UTR, confirming the interaction between NAT10 and this region. We have updated the text with more explanation of how this assay proves that NAT10 bound to the 3'UTR (line 270).

Comment 10b:

There also seem to be a discrepancy between the text, which suggest this is an acRIP-PCR, and the legend, which states it as being a NAT10 RIP-qPCR.

Response: We are very sorry for this mistake. We acknowledge that the term "acRIP-PCR" used in line 251 (new line 269) was incorrect. In fact, during the RIP-PCR experiment, we utilized the NAT10 antibody (Abcam, ab194297) to investigate whether NAT10 binds to Myc mRNA. We have corrected this error in the manuscript to accurately reflect that the experiment was an Anti-NAT10 antibody-based RIP-PCR, rather than acRIP-PCR (lines 270, 824).

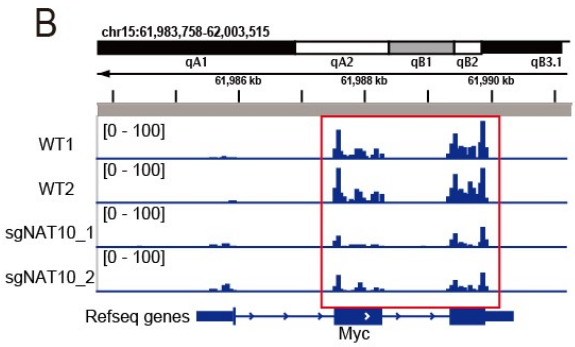
Comment 11:

- In lines 255-7, the authors claim that “Overall, these results demonstrate that NAT10 promotes the mRNA stability and translation efficiency of MYC via ac4C modification, and the ac4C peak in the 3’UTR region is responsible for mRNA stability”. What is the evidence for promotion of translation efficiency of Myc mRNA by NAT10? This claim needs supporting data, including the use of polysome profiling/RT-PCR.

Response: Thanks for your careful observation and valuable suggestion. We

acknowledge the lack of sufficient evidence in our original submission to support the claim regarding the promotion of MYC mRNA translation efficiency by NAT10. To address this, we conducted additional experiments, including Ribosome Profiling Sequencing (Ribo-seq) and RNA-seq analyses (GSE285518), to investigate the impact of NAT10 deletion on gene expression and translation efficiency.

Our results revealed that NAT10 deficiency leads to a significant reduction in both the mRNA levels and the translation efficiency of MYC (Response Figure 17, Updated Fig. S5B). These findings provided robust evidence supporting the role of NAT10 in regulating MYC mRNA translation efficiency. The revised manuscript now includes these data to substantiate our claims (Line 276, Fig. S5B).



Response Figure 17: (B) The peaks of myc in WT and sgNAT10 TC1 cells from Ribo-seq data were visualized using the IGV software.

Comment 12:

- It is quite surprising that overexpression of either Myc or CDK2 fully restores the phenotypes associated with NAT10 knockout back to those of the parental cells (e.g., Fig. 6D). Does this mean that all oncogenic functions of NAT10 are solely due to the upregulation of MYC and the subsequent upregulation of CDK2 and that no other target mRNA of NAT10, aside from Myc, nor any downstream targets of MYC besides CDK2, contribute to the observed phenotype? This is a critical point to address, as it appears that other targets of NAT10 identified in this study (e.g., Fig. 5D) also affect the expression of key proinflammatory cytokines (e.g., GIGYF1; PMID: 39018414).

Response: Thank you very for your careful and rigorous comments. Your question is very insightful, and we initially found it puzzling as well. After thorough consideration and discussion, we have identified two key factors that we believe

contribute to the observed phenomenon you mentioned:

(1) Restoration of Phenotypes by Overexpression of Myc and CDK2:

Our experiments demonstrated that NAT10 regulates the expression of key downstream targets, such as Myc and CDK2. Consequently, overexpressing these proteins can partially restore the phenotypes associated with NAT10 knockout. This recovery is likely driven by the direct actions of Myc and CDK2, both of which are known to promote tumor cell proliferation and suppress immune responses. Therefore, reintroducing Myc and CDK2 into the knockout cells likely reinstates functions that were lost upon NAT10 deletion.

(2) Overexpression of Myc and CDK2 in Stable Cell Lines:

It is important to note that in our rescue experiments, we employed stable cell lines expressing either Myc or CDK2, with plasmids containing GFP for selection. Flow cytometry was used to isolate cells with high GFP expression, indicating that these rescued cells exhibited elevated levels of Myc or CDK2 compared to WT cells. This overexpression likely surpasses the physiological levels observed in WT cells, which may account for the complete restoration of the phenotypes. Given that both Myc and CDK2 possess oncogenic properties, their overexpression could counteract the effects of NAT10 knockout by promoting cell proliferation and suppressing immune responses.

Taken together, these two factors provide a reasonable explanation for why the overexpression of Myc and CDK2 restores specific phenotypes observed in sgNAT10 cancer cells. First, NAT10 regulates the expression of these genes, and their reintroduction partially reverses the phenotypic alterations caused by NAT10 deficiency. Second, Myc and CDK2 inherently possess pro-proliferative and immunosuppressive properties, and their overexpression in sgNAT10 cancer cells further facilitates the restoration of these phenotypes. However, we acknowledge your concern regarding other potential downstream targets of NAT10. While Myc and CDK2 are central to our findings, we recognize that other NAT10 targets, such as GIGYF1, as you mentioned, may also contribute to the observed phenotypes, particularly in the context of proinflammatory cytokine regulation. We have addressed this point in our revised manuscript and will further elaborate on these additional pathways (lines 551-559, page 19).

We hope this explanation clarifies the reasoning behind our findings. Your thoughtful question has prompted us to deepen our understanding of the interactions between

NAT10, Myc, CDK2, and other potential targets, and we are grateful for the opportunity to refine our interpretation of these mechanisms.

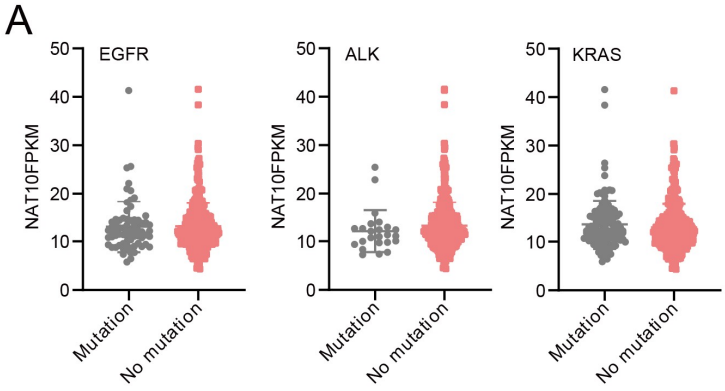
Thank you once again for your valuable input.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):
The authors have adequately addressed most of the concerns raised by the reviewers. However, there is still room for improvement regarding the clinical relevance of the study.
1.For lung adenocarcinoma (LUAD), the prognostic impact of EGFR or other driver gene mutations and the use of tyrosine kinase inhibitors (TKIs) is substantial. The authors should take this into account when analyzing the LUAD clinical data.

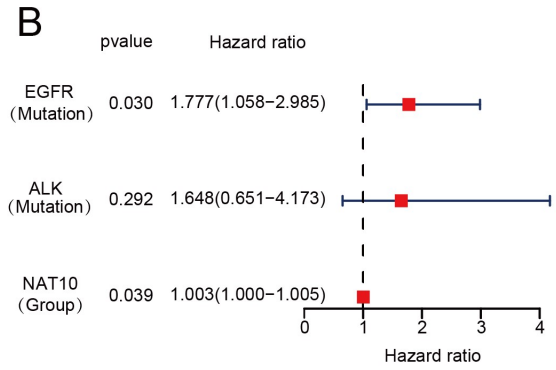
Response: Thank for very much for your overall positive comments. We appreciate your valuable concern about taking the driver gene mutations into account when analyzing the LUAD clinical data. Therefore, we reviewed and checked the mutation data of our lung cancer cohort. Unfortunately, we found that only 6 patients underwent EGFR mutation testing, with just one patient harboring the EGFR L858R mutation. The main reason is that all patients enrolled in the study were from year 2014–2015. These ten-years-before patients were suitable for survival analysis in our study; however, at that time, due to limited conditions, fewer people underwent mutation testing, and targeted therapies. As for other important driver genes in LUAD, such as ALK and KRAS, none of the patients had been tested for these mutations. We included these new clinical data in the new version (new supplementary table1).

Moreover, to further address your concern about the clinical data, we also reviewed the driver mutation data in TCGA-LUAD cohort. Patients were stratified into mutated and non-mutated groups based on mutation status of EGFR or ALK, regardless of mutation types. NAT10 expression levels were compared between the two groups. The results showed that no significant difference was found between the two groups (Response Figure 1A).



(A) To comprehensively evaluate the effect of gene mutations on the prognostic impact of NAT10 in LUAD, we retrieved EGFR, ALK gene mutation data from TCGA-LUAD cohort. For the relationship between NAT10 expression and gene mutations, the driver mutations, variants of uncertain significance (VUS), frame mutations, missense mutations, splice-site mutations, and truncating mutations were classified as mutations; all other cases were considered without mutation.

Additionally, we conducted a multivariate COX analysis of EGFR and ALK gene mutations as well as the expression level of NAT10. Consistent with our anticipations, NAT10 was demonstrated to play a role in facilitating the progression of the disease in patients with LUAD when taking into account the gene mutations (Response Figure 1B). We included these results and clarification in the new version of our manuscript (lines 119-123, Fig. S1E and S1F).



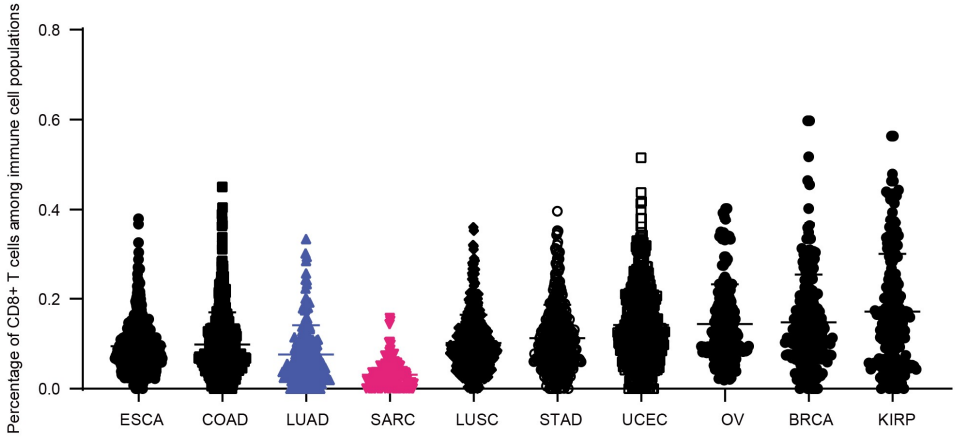
(B) For the multivariate COX analysis, we utilized “TCGA-biolink” package to obtain survival and status data for TCGA-LUAD cohort. Multivariate COX regression analysis was conducted using the "survival" package, with the confidence interval for the hazard ratio (HR) set at 95%.

2. The authors state that the use of a murine fibrosarcoma syngeneic cell line is intended to model "cold tumors." However, this approach has two key weaknesses: (1) there is no comparative analysis across multiple cancer types to support which tumors are "cold" or "hot," and (2) human sarcomas represent a highly heterogeneous group of diseases characterized by distinct fusion genes as drivers, which differ significantly from the syngeneic fibrosarcoma model used in this study. Incorporating analyses of human fibrosarcoma data and immune cell infiltration across multiple cancer types would improve the clinical relevance of the study.

Response: Thank you for your valuable suggestion. Tumors with particularly low densities of tumor-infiltrating T lymphocytes are classified as "cold tumors," whereas "hot tumors" exhibit a higher density of effector T cell infiltration and generally respond more robustly to immune checkpoint inhibitors. According to the literature,

lung cancer (PMID:37592367) and fibrosarcoma (PMID: 34277839) have been identified as immuno-cold tumors.

Additionally, to further substantiate our classification, we analyzed TCGA data to assess the proportion of CD8+ T cells in various tumor types. Specifically, we utilized the “TCGA-biolink” package to obtain gene expression data across pan-cancer cohorts. For immune cell infiltration analysis, we employed the “CIBERSORT” algorithm to quantify the proportions of 10 immune cell types, including CD8+ T cells, across different TCGA tumor datasets. Our results indicate that the LUAD and SARC we used in this study exhibit a relatively low percentage of CD8+ T cells, supporting its classification as a "cold tumor" (Response Figure 2).



Moreover, we appreciate your comment about the sarcoma classification. We have explored several databases; however, there is currently a lack of comprehensive classification for sarcomas, making it challenging to conduct an in-depth analysis of their clinical relevance. We recognize the importance of this aspect and will continue to monitor advancements in this area to enhance the clinical applicability of our research.

Reviewer #2 (Remarks to the Author):
The author has addressed my concerns, and I have no further questions.

Response: Thank you very much for your overall positive comment.

Reviewer #3 (Remarks to the Author):

The authors have made commendable efforts and have largely succeeded in addressing my comments on the first version of this manuscript. However, a few outstanding issues still need to be resolved

1- In response to my original comment "In line 251, the authors claim that "the results of acRIP-PCR also confirmed that NAT10 bound to the 3'UTR of MYC (Fig. 5I)". It is not clear how this assay proves that NAT10 bound to the 3'UTR" the authors replied that "In Fig. 5I, an anti-NAT10 antibody-based RIP-PCR instead of acRIP-PCR (We are sorry for the mistake) experiment was conducted using primers specifically targeting the MYC 3'UTR, confirming the interaction between NAT10 and this region. We have updated the text with more explanation of how this assay proves that NAT10 bound to the 3'UTR (line 270)."

However, this response still does not address how the assay definitively proves binding to the 3′ UTR. The authors state that they used primers specific to the 3′ UTR, which only demonstrates that the pulled-down mRNA contains the 3′ UTR. It does not confirm that NAT10 binds specifically to the 3′ UTR. NAT10 could potentially bind to the CDS or 3′ UTR, and the primers would still amplify the 3′ UTR sequence.

In my opinion, the experimental design is flawed and cannot conclusively demonstrate binding to the 3′ UTR. To address this issue, the authors would need to use a control mRNA where the 3′ UTR is either mutated to disrupt the NAT10 binding site or entirely absent. If the hypothesis is correct, the RIP-PCR should only yield a positive result for the full-length cDNA and not for the mutant or truncated mRNA.

Response: Thank you for your valuable comments and for pointing out the experimental limitations in Figure 5I. You are correct in highlighting this flaw in our design, and we sincerely appreciate your critical insight. We acknowledge our oversight and have taken your suggestion into account by conducting additional experiments to address this issue.

To validate the specific binding of NAT10 to the 3′ UTR of Myc mRNA, we constructed a mutant full-length MYC gene sequence with targeted mutations in the 3′UTR to disrupt the NAT10 binding site (Figure 5G) and cloned it into the VP64-GFP plasmid. We then transfected 293T cells with either WT MYC-V64 or Mut MYC-V64, along with the NAT10-V64 plasmid, and performed an anti-NAT10 antibody-based RIP-PCR experiment. DNA gel electrophoresis demonstrated that the NAT10 antibody successfully pulled down WT Myc mRNA, while Myc mRNA containing the mutated 3′UTR was not enriched. These findings suggested that NAT10 predominantly binds to the 3′ UTR of Myc mRNA.

We included these results and clarification in the new version of our manuscript

(lines 273-277, Fig. 5I).

2- Regarding the claim of translational regulation of MYC mRNA by NAT10, the authors conducted a Ribo-Seq assay, which is appropriate and demonstrates reduced footprint levels on MYC mRNA. However, since the KO cells also exhibit lower MYC mRNA expression compared to the WT, the conclusion that the “translation efficiency” of MYC mRNA is reduced is not entirely accurate. I recommend the authors revise the manuscript to indicate that both mRNA expression and footprint levels are downregulated, leading to an overall decrease in protein expression and avoid the term “translation efficiency”, which would likely not be appropriate for the observed changes. Additionally, I would like to draw the authors' attention to the increased footprint levels in the 5′ UTR of MYC mRNA in KO cells. This observation could be related to activation of the ISR pathway, which, while beyond the scope of this manuscript, may be worth exploring in future studies.

Response: Thank you for your constructive comments. We have revised the manuscript as your suggestion. Specifically, we have modified the text to: 'We also performed Ribo-seq analysis to investigate whether NAT10 regulates the translation of MYC mRNA (Supplementary Data 7). Our findings showed a reduction in footprint levels on MYC mRNA (Fig. S5B), indicating an overall decrease in MYC protein expression.' This revision clarifies that both MYC mRNA expression and footprint levels are downregulated, leading to an overall decrease in protein expression, as you suggested. We also appreciate your comment on the increased footprint levels in the 5′ UTR of MYC mRNA in KO cells, which could indeed be related to the activation of the ISR pathway. While we did not explore this in the current study, we acknowledge its potential relevance for future research. Based on your suggestion, we have revised the manuscript to clarify this (line 281-284).

3. In response to my last comment on the 1st version of the manuscript being overly focused on MYC and CDK2, the authors have expanded on their explanation regarding how restoring MYC or CDK2 expression rectified the observed phenotypes. While I do not question their observations or explanation, I believe it would be appropriate to acknowledge in the Discussion section that the MYC-CDK2 axis is likely only one of the downstream mechanisms through which NAT10 regulates the phenotype and that there are likely other potentially significant downstream factors that were not explored in detail.

Response: Thank you for your thoughtful comment. We have revised the Discussion section to clarify that the MYC-CDK2 axis is not the only downstream pathway through which NAT10 regulates the phenotype. We also acknowledge that other pathways may be affected, though these were not explored in detail in the current study. We have addressed this limitation in the revised manuscript (lines 562-565).

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the raised concerns, and I am satisfied with their response and the revised manuscript.

Response: Thank you very much for your overall positive comment.

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns regarding the previous version of the manuscript, and I have no further questions.

Response: Thank you very much for your overall positive comment.