nature portfolio

Peer Review File

m6A modification regulates cell proliferation via reprogramming the balance between glycolysis and pentose phosphate pathway

Corresponding Author: Dr Guan-Zheng Luo

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors define an important role of m6A modification regulates cell proliferation via reprogramming the balance between glycolysis and pentose phosphate pathway. The authors revealed that the depletion of m6A on TIGAR mRNA led to increased expression, subsequently inhibiting glycolysis while promoting the pentose phosphate pathway. They applied genome-wide CRISPR-Cas9 screen to identify genes involved in cell proliferation. The manuscript would be strengthened by addressing the following comments.

Major comments:
1. The authors showed that m6A inhibitor STM2457 inhibits cell proliferation by reprogramming the balance between glycolysis and PPP pathway. However, as many studies reported, the PPP pathway plays a critical role in maintaining cancer cell growth. The authors showed that STM2457 treatment leads to less TIGAR degradation and enhanced PPP pathway while inhibiting cancer cell proliferation. The authors explain this contradiction.
2. As same mentioned in Major points #1, G6PD generally considered to play important roles in supporting cancer cell growth. However, the authors showed the adverse effects in LIHC, although other groups have reported the completely different results in LIHC.

Minor points:
1. Please apply superscript formatting to the 6 in m6A.
2. The authors should confirm some experiments in METTL3 or METTL14 knockdown cell lines to confirm the STM2457 treatment results.
3. As shown in figure 1B, why there were an increase in m6A ration after 16 hours treatment compared to 8 hours treatment?
4. The authors should include the control group in Figure 6E.

Reviewer #2

(Remarks to the Author)
In their manuscript, Luo et al. showed that reduced m6A levels via STM2457 results in reduced cell proliferation. They showed that this effect is due to prolonged S phase. TIGAR was shown to be a downstream effector for the associated phenotype. Through metabolomics and genome-wide screening, authors showed that altered metabolic pathways are important to explain the reduced cell proliferation. By elegant validation of the screen, they focused on the role of G6PD and showed rescue of the phenotype with STM2457. Results were clearly communicated, figures are high quality and especially the genome-wide CRISPR-Cas9 screen would be used as a resource for further research in the field. I do not have any specific comments to improve the current state of the manuscript.

Reviewer #3

(Remarks to the Author)
In the manuscript by Jian-Fei Xi et al., the researchers studied the effect of low m6A levels on cell proliferation mainly based on the model of HEK293T cells. They revealed that the depletion of m6A on metabolism target TIGAR mRNA led to increased expression, subsequently inhibiting glycolysis while promoting the pentose phosphate pathway (PPP). Moreover, they performed a genome-wide CRISPR-Cas9 screen in 293T cell at normal and low m6A condition and identified numerous candidates that possibly sensitize the responsiveness to cell proliferation through m6A suppression.

The whole study draws general conclusion on the dependency of m6A on cell proliferation and cellular mentalism. However, the metabolic reprogramming is one of hallmark of cancer progression. I hardly believe the knowledge from the 293T cell model could represent the complicated tumor cells. Moreover, the study is lack of downstream functional validation for many potentially interesting targets from their genetic screen. For example, whether the change of expression level in TIGAR or GP6D could indeed explain the glycolysis inhibition and PPP activation? With that, please see my major comments below:

1) In Fig 1, in my opinion, there is no novel finding. Although HEK293T cells is responsive to the treatment of METTL3 inhibitor STM2457, it is not an appreciate model to study cancer metabolic reprogramming. Moreover, what about the cellular effect by simply knocking out METTL3 in HEK293 cell? I could understand HEK293T cell is a simple model for genetic screen, but for sure the downstream targets and metabolism effect needs to be validated in more cellular context. For example, the authors show some data on HuH7 cell or Hela cell. It would be ideal to pick up some sensitive or resistant cell lines from Fig 1A to compare side-by side.

2) In Fig 2, the authors focused on one main target by their integrative analysis. However, what is the effect of TIGAR in cellular proliferation of HEK293T with or without STM treatment? A functional rescue experiment is needed.

3) In Fig 2 and Fig 3, again the authors performed transcriptome and metabolite profiling in STM treated HEK293T cells. Is this phenomenon specific to 293T cells or in general? This is the key unsolved question based on this study.

4) In Fig 4, the whole CRISPR/Cas9 genetic screen with/wo STM treatment is informative, however the analysis and functional validation is superficial. I think it would be meaningful to give a relative sensitivity score of candidate genes with/wo STM. It is important to know how those candidates affect the IC50 of STM in HEK293T cell line, and more other tumor cell models. Moreover, many candidates are lack of expression validation or even knockout efficiency validation.

5) It is hard for readers to switch to the cell cycle arrest by G6PD knockout in Fig 5. The authors identified that knockout of G6PD might cause drug resistance. This is interesting finding but lack of thorough functional validation. I am surprised they switched to validate the S phase arrest.

6) Again, in Fig 6, they showed tons of correlation data from TCGA but providing piece of functional work by a second activator of G6PD. What is cellular consequence such as cell proliferation by the knockout or overexpression of G6PD in METT3L null or suppressed context. And this also could be tested in more tumor cell context.

Reviewer #4

(Remarks to the Author)
In this manuscript, Xi and his/her colleagues use genetical and pharmacological approaches to evaluate the function of m6A modifications in cell proliferation. By employing several tumor cell lines, and HEK293T as well, they found that: (1) low-m6A levels delay cell proliferation (strong evidence); (2) low-m6A levels elevate TIGAR mRNA stability (strong evidence); (3) low-m6A levels lead to reduced glycolysis, but increased pentose phosphate pathway (weak and misleading evidence); (4) Parallel Genome-wide CRISPR identify genetic effectors of cellular sensitivity to m6A modification (strong evidence)；(5) low-m6A levels lead to S phase arrest (Interesting finding but with very weak mechanistic support); (6) low-m6A levels plus G6PD activation potentially has cell growth arrest benefit, but they used non-tumor cell line HEK293T in this experiment. In general, the authors made several interesting observations, but the mechanistic study has much room to be improved.

Major concerns:
(1) To support the metabolic reprogramming from glycolysis to pentose phosphate way, the authors have to perform the glucose tracing experiment, rather than only using unlabeled metabolite massspec.
(2) Why reduced ROS production lead to CDK2 deactivation? Any experimental explanation?

Minor concern:
(1) Figure 3C: Mislabeled? Should the yellow bar represent the DMSO group? BTW, “7” in STM2457 is also missing.
(2) Results section and figure legends: All the titles are present in a very vague way. The reader can’t absorb any key information from the titles. The authors should change them to be more accurate and more conclusive.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns. I appreciate their efforts to strengthen their works and have no more questions. I recommend the acceptance in Communications Biology.

Reviewer #4

(Remarks to the Author)
The authors have nicely addressed all my concerns. Therefore, I recommend the publication of this revised manuscript.

Point-by-point response to reviewer comments

Summary

We are very grateful for the constructive comments and suggestions from four reviewers. We revised the manuscript and updated figures accordingly. We believe that all the concerns and questions raised by the reviewers have now been well-addressed. Please check the following point-by-point response. Our replies are highlighted, and the modified text/figures in the manuscript are also marked.

Reviewer #1 (Remarks to the Author):
In this manuscript, the authors define an important role of m^6A modification regulates cell proliferation via reprogramming the balance between glycolysis and pentose phosphate pathway. The authors revealed that the depletion of m^6A on TIGAR mRNA led to increased expression, subsequently inhibiting glycolysis while promoting the pentose phosphate pathway. They applied genome-wide CRISPR-Cas9 screen to identify genes involved in cell proliferation. The manuscript would be strengthened by addressing the following comments.

Response: We sincerely appreciate your positive comments and valuable suggestions, which have helped us significantly strengthen this work. We have carefully considered each point and have made revisions accordingly. Specifically, we have addressed your concerns regarding the role of the pentose phosphate pathway (PPP) in cancer cell growth and its modulation by m^6A modification (Major Comment #1), clarifying that while the PPP can contribute to nucleotide biosynthesis, the reduction in glycolytic flux appears to be the primary driver of growth inhibition in our model. We also refined the analysis of G6PD in liver hepatocellular carcinoma (LIHC) to reconcile our results with existing literature (Major Comment #2), providing a more nuanced perspective on G6PD's function in the context of m^6A modification.

In addition, we addressed other minor points, including correcting the m^6A formatting throughout the manuscript (Minor Point #1), performing validation experiments in METTL3 knockdown cell lines (Extended Figure 1, Minor Point #2), providing a detailed explanation for the m^6A ratio changes observed in Supplementary Figure 1, and adding the control group to Figure 6E. We believe these revisions have significantly enhanced the clarity of our study and directly address your valuable feedback.

Major comments:
1. The authors showed that m6A inhibitor STM2457 inhibits cell proliferation by reprogramming the balance between glycolysis and PPP pathway. However, as many studies reported, the PPP pathway plays a critical role in maintaining cancer cell growth. The authors showed that STM2457 treatment leads to less TIGAR degradation and enhanced PPP pathway while inhibiting cancer cell proliferation. The authors explain this contradiction.

Response: Thank you for raising this important point. Our findings demonstrate that STM2457 inhibits cell proliferation despite an observed increase in PPP activity. This seemingly paradoxical result can be explained by the dominant role of glycolysis in the cancer cells used in our study. By increasing TIGAR expression, STM2457 shifts the metabolic flux away from glycolysis and towards the PPP. While the PPP can contribute to nucleotide biosynthesis[1], the reduction in glycolytic flux appears to be the primary driver of the observed growth inhibition[2-5]. This is supported by the finding that G6PD deficiency has no effects on the cell growth and progression of certain types of tumors[6,7]. Furthermore, G6PD deficient tumor cells proliferate normally via the compensatory malic enzyme 1 and isocitrate dehydrogenase 1 flux[8].

We acknowledge that the PPP is essential for the growth of certain cancer types. However, our data suggest that glycolysis plays a more prominent role in supporting proliferation. We have revised the Discussion to clarify this point and discuss the context-dependent nature of the PPP's role in cancer cell growth, with a focus on the critical balance between glycolysis and PPP.

2. As same mentioned in Major points #1, G6PD generally considered to play important roles in supporting cancer cell growth. However, the authors showed the adverse effects in LIHC, although other groups have reported the completely different results in LIHC.

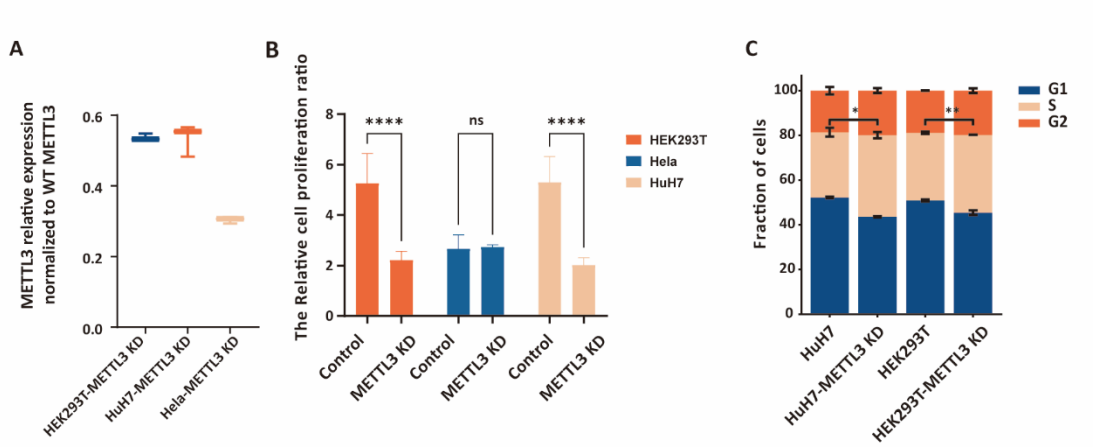
Response: Thank you for highlighting the complex role of G6PD in LIHC. We acknowledge that previous studies have reported the important role of G6PD in supporting cancer cell growth. However, our findings, which show a better prognosis for patients with higher G6PD expression but low METTL3 expression, suggest that m^{6}A modification may modulate the role of G6PD in this context. This observation supports our assertion that m^{6}A modification influences tumor cell proliferation by regulating glycolytic pathways. We have refined the corresponding section to clarify this point.

Minor points:
1. Please apply superscript formatting to the 6 in m^{6}A.

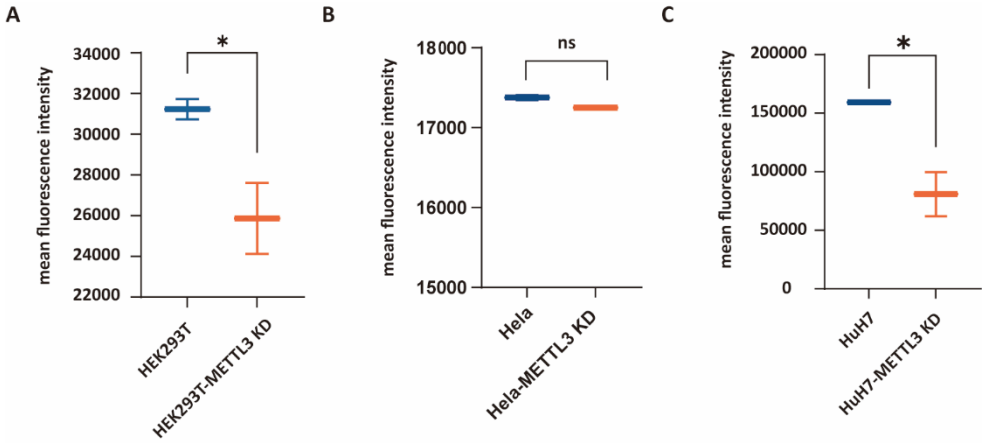
Response: Thank you for this suggestion and we have corrected the formatting of m^{6}A throughout the manuscript.

2. The authors should confirm some experiments in METTL3 or METTL14 knockdown cell lines to confirm the STM2457 treatment results.

Response: Thank you for raising this valuable suggestion. We conducted METTL3 shRNA knockdown in HEK293T, HuH7, and HeLa cell lines (Extended Figure 1A). METTL3 knockdown significantly inhibited proliferation of HEK293T and HuH7 cells and resulted in similar cell cycle changes as observed with STM2457 treatment (Extended Figures 1B, 1C). ROS levels were also decreased in HEK293T and HuH7 cells upon METTL3 knockdown (Extended Figure 2). These results, now included in the revised manuscript, support our findings with STM2457 treatment and further strengthen the role of m^{6}A in regulating cell proliferation and ROS levels.



Extended Figure 1. **Inhibition of cell proliferation by METTL3 knockdown in multiple cell lines. A.** Efficiency of METTL3 shRNA knockdown in HEK293T, HuH7, and HeLa cell lines. **B**. Relative cell proliferation ratio following METTL3 knockdown. **C**. Cell cycle analysis in METTL3 knockdown HEK293T and HuH7 cells.



Extended Figure 2. Impact of METTL3 knockdown on cellular ROS levels across multiple cell lines. A. ROS level in HEK293T cells with METTL3 knockdown. The mean fluorescence intensity indicates the ROS level. **B**. ROS level in HeLa cells with METTL3 knockdown. **C**. ROS level in HuH7 cells with METTL3 knockdown.

3. As shown in figure 1B, why there were an increase in m^{6}A ration after 16 hours treatment compared to 8 hours treatment?

Response: Thank you for this observation. We have carefully reviewed the experimental data with repeats and did not identify any apparent errors (Supplementary Figure 1). We speculate that the increase in the m^{6}A ratio after 16 hours compared to 8 hours in Figure 1B could be attributed to the dynamic interplay between STM2457's inhibitory effect and cellular compensatory mechanisms. While STM2457 initially reduces m^{6}A levels, the cells may partially restore m^{6}A modification through as yet undefined compensatory pathways[12,13]. The subsequent decrease in m^{6}A levels at later time points suggests that these compensatory mechanisms may be transient. Further investigation into the precise dynamics of m^{6}A regulation in response to STM2457 is warranted.

4. The authors should include the control group in Figure 6E.

Response: Thank you for this suggestion. We have added the control group to Figure 6E.

Reviewer #2 (Remarks to the Author):

In their manuscript, Luo et al. showed that reduced m^{6}A levels via STM2457 results in reduced cell proliferation. They showed that this effect is due to prolonged S phase. TIGAR was shown to be a downstream effector for the associated phenotype. Through metabolomics and genome-wide screening, authors showed that altered metabolic pathways are important to explain the reduced cell proliferation. By elegant validation of the screen, they focused on the role of G6PD and showed rescue of the phenotype with STM2457. Results were clearly communicated, figures are high quality and especially the genome-wide CRISPR-Cas9 screen would be used as a resource for further research in the field. I do not have any specific comments to improve the current state of the manuscript.

Response: We sincerely thank this reviewer for their positive assessment of our manuscript and appreciate their recognition of the potential value of our CRISPR screen data as a resource for future research.

Reviewer #3 (Remarks to the Author):

In the manuscript by Jian-Fei Xi et al., the researchers studied the effect of low m^{6}A levels on cell proliferation mainly based on the model of HEK293T cells. They revealed that the depletion of m^{6}A on metabolism target TIGAR mRNA led to increased expression, subsequently inhibiting glycolysis while promoting the pentose phosphate pathway (PPP). Moreover, they performed a genome-wide CRISPR-Cas9 screen in 293T cell at normal and low m^{6}A condition and identified numerous candidates that possibly sensitize the responsiveness to cell proliferation through m^{6}A suppression.

The whole study draws general conclusion on the dependency of m^{6}A on cell proliferation and cellular mentalism. However, the metabolic reprogramming is one of hallmark of cancer progression. I hardly believe the knowledge from the 293T cell model could represent the complicated tumor cells. Moreover, the study is lack of downstream functional validation for many potentially interesting targets from their genetic screen. For example, whether the change of expression level in TIGAR or GP6D could indeed explain the glycolysis inhibition and PPP activation? With that, please see my major comments below:

Response: We highly appreciate your positive comments and valuable suggestions. In response to your insightful feedback, we have significantly strengthened the manuscript by expanding our study to include HuH7 and HeLa cell lines, providing a more comprehensive understanding of m^{6}A's role in cell proliferation and metabolism across different cancer cell types. We also performed functional validation of key targets, including TIGAR and G6PD, and demonstrated consistent metabolic reprogramming in multiple cell lines. We summarized our revisions as below:

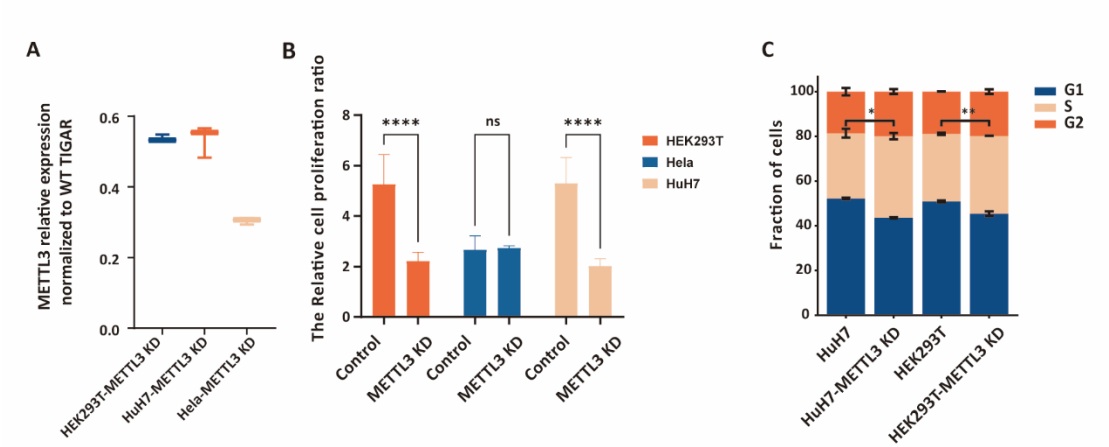
1. Broadening Cellular Context. We acknowledge your concern about the reliance on the

HEK293T cell model and have expanded our studies to include HuH7 and HeLa cell lines. This additional data provides a more comprehensive understanding of the effects of METTL3 inhibition across different cancer cell types.
2. METTL3 Knockdown and Cell Proliferation Analysis. We have performed METTL3 shRNA knockdown in HEK293T, HuH7, and HeLa cell lines and assessed the proliferation of these cell lines. We found that METTL3 knockdown significantly inhibited cell proliferation in HEK293T and HuH7 cells, while the effect in HeLa cells was less pronounced.
3. Cell Cycle and ROS Level Analysis. We analyzed the cell cycle in the three METTL3 knockdown cell lines and observed S phase inhibition in HuH7 and HEK293T cells. Additionally, we measured intracellular ROS levels and found a significant decrease in ROS levels in HEK293T and HuH7 cells following METTL3 knockdown.
4. Functional Validation of TIGAR. We performed TIGAR knockout in HEK293T, HuH7, and HeLa cells and assessed the cell proliferation rates. We found that TIGAR knockout significantly inhibited cell proliferation in all three cell lines, consistent with the observed effects of STM2457 treatment.
5. Metabolic Reprogramming and Generalizability. We performed metabolic profiling in HuH7 and HEK293T cells following METTL3 knockdown. We observed a significant enhancement of the PPP in both cell lines, indicating that the metabolic reprogramming is not unique to HEK293T cells.
6. Validation of CRISPR/Cas9 Genetic Screen. We have provided additional expression validation for candidate genes from our genetic screen using RT-qPCR. We have also conducted knockout and rescue experiments for G6PD in HEK293T and HuH7 cells, highlighting its role in proliferation under low m^6A conditions.
7. Cellular Function of G6PD. We have assessed the cellular consequences of G6PD knockout or overexpression in METTL3-suppressed cells. Our findings demonstrate that G6PD is crucial for cell proliferation under low m^6A conditions and its manipulation can significantly influence the proliferation rates of these cells.
We believe these revisions and additional experiments address your concerns and provide a more thorough understanding of the role of m^6A modification in cell proliferation and metabolism.

1. In Fig 1, in my opinion, there is no novel finding. Although HEK293T cells is responsive to the treatment of METTL3 inhibitor STM2457, it is not an appreciate model to study cancer metabolic reprogramming. Moreover, what about the cellular effect by simply knocking out METTL3 in HEK293 cell? I could understand HEK293T cell is a simple model for genetic screen, but for sure the downstream targets and metabolism effect needs to be validated in more cellular context. For example, the authors show some data on HuH7 cell or Hela cell. It would be ideal to pick up some sensitive or resistant cell lines from Fig 1A to compare side-by side.

Response: Thank you for pointing out the limitations of relying solely on HEK293T cells for studying cancer metabolic reprogramming. We have expanded our analyses to include HuH7 and HeLa cell lines, chosen for their varying sensitivities to METTL3 inhibition observed in the initial screen. We now present data on METTL3 knockdown (Extended

Figure 1A) in all three cell lines, including proliferation assays (Extended Figure 1B), cell cycle analysis (Extended Figure 1C), and ROS level measurements (Extended Figure 2). These data demonstrate consistent effects of METTL3 modulation across different cellular contexts, strengthening the generalizability of our findings.



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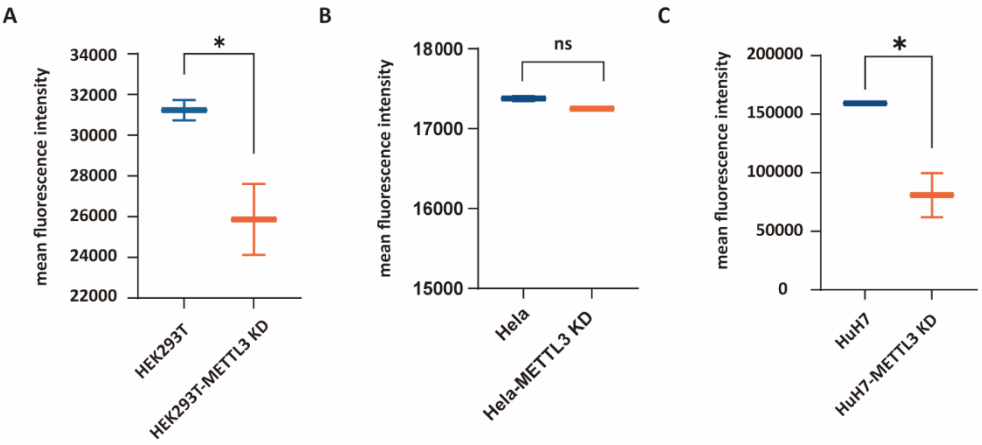
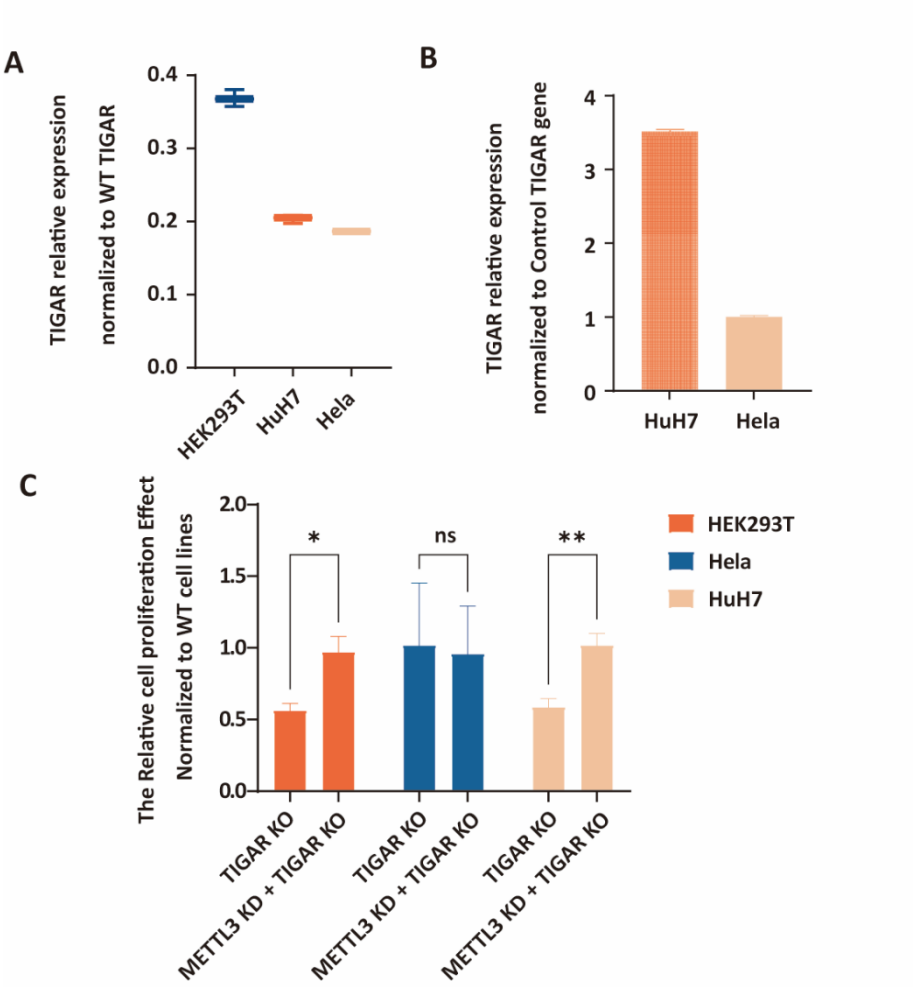


Extended Figure 2. Impact of METTL3 knockdown on cellular ROS levels across multiple cell lines. A. ROS level in HEK293T cells with METTL3 knockdown. The mean fluorescence intensity indicates the ROS level. **B**. ROS level in HeLa cells with METTL3 knockdown. **C**. ROS level in HuH7 cells with METTL3 knockdown

2. In Fig 2, the authors focused on one main target by their integrative analysis. However, what is the effect of TIGAR in cellular proliferation of HEK293T with or without STM treatment? A functional rescue experiment is needed.

Response: Thank you for your insightful comments and suggestions regarding the role of TIGAR in cellular proliferation. Your feedback has prompted us to conduct additional functional experiments to further elucidate the role of TIGAR. We examined TIGAR expression changes upon METTL3 knockdown, revealing significant upregulation in HuH7

but not in HeLa cells (Extended Figure 3B), suggesting a cell type-specific effect. We further assessed the proliferation phenotypes in cells with METTL3 knockdown and METTL3/TIGAR co-suppression (Extended Figure 3A). As shown in Extended Figure 3C, co- suppression of TIGAR rescued the proliferation defect caused by METTL3 knockdown, restoring proliferation rates to wild-type levels.

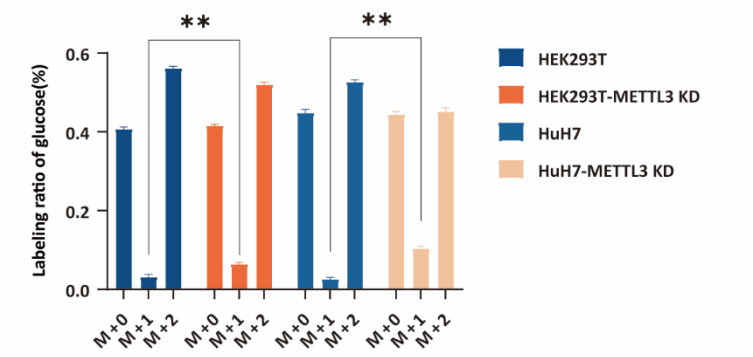


Extended Figure 3. TIGAR mediates the anti-proliferative effects of m^{6}A reduction. **A**. TIGAR mRNA expression after CRISPR-Cas9 KO in three cell lines. **B**. Effect of METTL3 shRNA KD on TIGAR gene expression in HuH7 and HeLa cells. Knockdown of METTL3 leads to an increase in TIGAR gene expression in HuH7 cell lines, as indicated by quantitative RT-qPCR analysis. **C**. Impact of TIGAR KO and METTL3 KD on cell proliferation. Comparative analysis of relative cell proliferation ratios in HEK293T, HuH7, and HeLa cell lines following TIGAR KO and METTL3 KD.

3. In Fig 2 and Fig 3, again the authors performed transcriptome and metabolite profiling in STM treated HEK293T cells. Is this phenomenon specific to 293T cells or in general? This is the key unsolved question based on this study.

Response: Thank you for raising this crucial question. To address the generalizability of our findings beyond HEK293T cells, we performed metabolic profiling in both HuH7 and HEK293T cells following METTL3 knockdown using ^{13}C-labeled glucose combined with

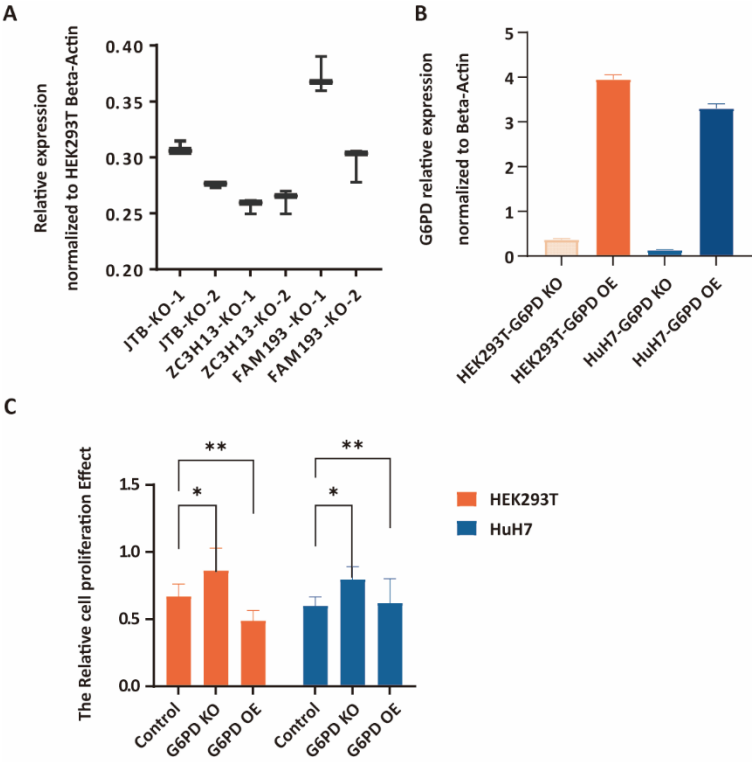
chromatography and mass spectrometry. The results showed a consistent enhancement of the PPP in both cell lines (Extended Figure 4), indicating that the observed metabolic reprogramming is not unique to HEK293T and is likely a more general effect of m^{6}A modulation[14].



Extended Figure 4. Isotopic tracing analysis of the pentose phosphate pathway in HuH7 and HEK293T cells following METTL3 knockdown. Cells were cultured in medium containing 13C-glucose. The relative abundance of different isotopologues of lactate (M+0, M+1, and M+2) is shown.

4. In Fig 4, the whole CRISPR/Cas9 genetic screen with/wo STM treatment is informative, however the analysis and functional validation is superficial. I think it would be meaningful to give a relative sensitivity score of candidate genes with/wo STM. It is important to know how those candidates affect the IC50 of STM in HEK293T cell line, and more other tumor cell models. Moreover, many candidates are lack of expression validation or even knockout efficiency validation.

Response: Thank you for this suggestion. We have provided additional RT-qPCR validation of candidate gene expression changes following CRISPR-Cas9 KO (Extended Figure 5A), Knockout efficiency for these genes was previously confirmed and is presented in Supplementary Figure 2C. We appreciate your interest in the biological functional of those candidate genes we identified. The original manuscript already includes relevant information on how the KO of specific genes affects the cell proliferation in a low m^{6}A condition (Figure 4F). To further explore their functional implications in other cells, we conducted KO and rescue experiments for G6PD in both HEK293T and HuH7 cells with METTL3 KD, demonstrating its critical role in proliferation under low m^{6}A conditions (Extended Figure 5B, 5C). We hope that these additional details and experiments address your concerns and provide a more comprehensive understanding of the genetic screen outcomes and their implications for cancer cell proliferation.



Extended Figure 5. Validation of candidate genes in METTL3 knockdown cell lines. **A.** RT-qPCR confirmation of KO efficiency of candidate genes. **B.** RT-qPCR validation of G6PD KO or overexpression in HEK293T and HuH7 cell lines with METTL3 KD. **C.** The effects of G6PD KO and overexpression for cell proliferation in HEK293T and HuH7 cells.

5. It is hard for readers to switch to the cell cycle arrest by G6PD knockout in Fig 5. The authors identified that knockout of G6PD might cause drug resistance. This is interesting finding but lack of thorough functional validation. I am surprised they switched to validate the S phase arrest.

Response: Thank you for your thoughtful feedback on the functional implication of G6PD in our study. We also believe that the potential role of G6PD in drug resistance would be one of the most exciting discovery. However, it is very difficult for our lab to carry out further in vivo experiments, and our primary focus in this study was to elucidate the mechanism by which G6PD knockout influences proliferation in the context of altered m^6A levels. Nevertheless, we have conducted knockout and overexpressing experiments for G6PD in both HEK293T and HuH7 cells. Our results indicate that G6PD plays a pivotal role in cell proliferation, and the cells with G6PD knockout resist the deficiency caused by low m^6A.

To investigate the underlying cause of the cellular phenotype, we examined the effect of reduced m^6A levels on cell proliferation and found that it leads to S phase arrest. G6PD has been identified to be essential in regulating intracellular oxidative stress levels. After identifying G6PD as a candidate gene, we measured the levels of reactive oxygen species (ROS) in the cells and found that reduced m^6A levels lead to a decrease in intracellular ROS levels. While excessive ROS levels can cause oxidative stress and metabolic disorders in cells, maintaining a certain level of ROS is crucial for cellular signaling.

Therefore, we carried out further analysis to show that a decrease in ROS levels indeed affects the phosphorylation levels of cell cycle protein CDK2, thereby influencing S phase arrest.

Our findings suggest that the modulation of ROS levels by changes in m^6A methylation status plays a significant role in cell cycle control, which is a novel aspect of our study. We hope that these additional details and clarifications address your concerns and provide a more comprehensive understanding of the role of G6PD in cellular responses to m^6A modulation and its implications for cell cycle regulation. Thank you again for your valuable feedback.

6. Again, in Fig 6, they showed tons of correlation data from TCGA but providing piece of functional work by a second activator of G6PD. What is cellular consequence such as cell proliferation by the knockout or overexpression of G6PD in METTL3 null or suppressed context. And this also could be tested in more tumor cell context.

Response: We appreciate your insightful comments. We totally agree that deeper studies are invaluable to understand the role of G6PD in cell proliferation and its potential as a therapeutic target. We believe that future experiments, building upon the foundation established in this study, will provide a more comprehensive understanding of the interplay between G6PD and METTL3 in cancer. In response to your suggestion, we conducted G6PD knockout and overexpression in HEK293T and HuH7 cells (Extended Figure 5B), demonstrating its critical role in proliferation under low m^6A conditions (Extended Figure 5C). Thank you again for your constructive feedback, which has been instrumental in enhancing the depth and breadth of our study.

Reviewer #4 (Remarks to the Author):
In this manuscript, Xi and his/her colleagues use genetical and pharmacological approaches to evaluate the function of m^6A modifications in cell proliferation. By employing several tumor cell lines, and HEK293T as well, they found that: (1) low-m^6A levels delay cell proliferation (strong evidence); (2) low-m^6A levels elevate TIGAR mRNA stability (strong evidence); (3) low-m^6A levels lead to reduced glycolysis, but increased pentose phosphate pathway (weak and misleading evidence); (4) Parallel Genome-wide CRISPR identify genetic effectors of cellular sensitivity to m^6A modification (strong evidence)；(5) low-m^6A levels lead to S phase arrest (Interesting finding but with very weak mechanistic support); (6) low-m^6A levels plus G6PD activation potentially has cell growth arrest benefit, but they used non-tumor cell line HEK293T in this experiment. In general, the authors made several interesting observations, but the mechanistic study has much room to be improved.

Response: We highly appreciate your positive comments and valuable suggestions. In response to your feedback, we have made several key revisions to strengthen the manuscript. We have conducted glucose tracing experiments using ^{13}C-labeled glucose combined with chromatography and mass spectrometry in both HuH7 and HEK293T cells following METTL3 knockdown. These experiments provide strong evidence that the metabolic reprogramming is not limited to HEK293T cells but is also observed in HuH7 cells, indicating a general mechanism across different cell types. We acknowledge your

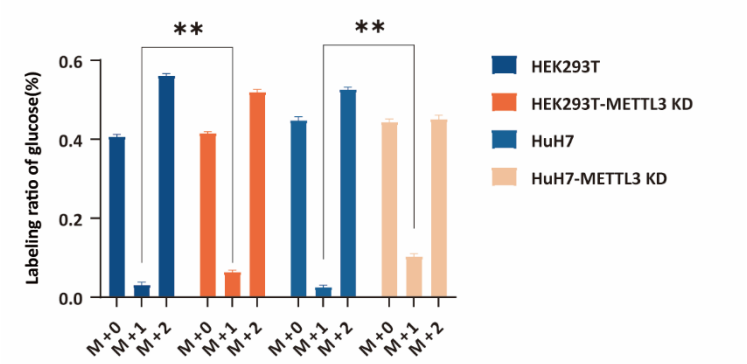
concern about the source of the information regarding ROS modulation of CDK2 activity. We have revised the manuscript to properly attribute this to the existing literature and to clarify that our results are consistent with these findings. In addition, we have corrected errors and misleading expression in text and figures.

We believe these revisions address your concerns and significantly enhance the clarity, depth, and scientific rigor of our study. We are grateful for your constructive feedback, which has been instrumental in refining our manuscript.

Major concerns:

1. To support the metabolic reprogramming from glycolysis to pentose phosphate way, the authors have to perform the glucose tracing experiment**,** rather than only using unlabeled metabolite massspec.

Response: We appreciate this important suggestion. We have performed glucose tracing experiments using ^{13}C-labeled glucose, combined with chromatography and mass spectrometry, in both HuH7 and HEK293T cells following METTL3 knockdown. Our results demonstrate that METTL3 KD significantly enhances the pentose phosphate pathway (PPP) in both cell lines, suggesting that the metabolic reprogramming is not limited to HEK293T cells (Extended Figure 4). These results also suggest that targeting m^{6}A modification could be a viable strategy to influence metabolic pathways in cancer cells, potentially impacting cancer cell proliferation and survival.



Extended Figure 4. Isotopic tracing analysis of the pentose phosphate pathway in HuH7 and HEK293T cells following METTL3 knockdown. Cells were cultured in medium containing 13C-glucose. The relative abundance of different isotopologues of lactate (M+0, M+1, and M+2) is shown.

2. Why reduced ROS production lead to CDK2 deactivation? Any experimental explanation?

Response: Thank you for this question. Our findings, which are supported by recent research, indicate that reactive oxygen species (ROS) play a pivotal role in regulating CDK2 activity, particularly through the oxidation of a conserved cysteine residue near the T-loop of CDK2[15]. This oxidation event is crucial as it prevents the binding of the T-loop phosphatase KAP, which would otherwise dephosphorylate and inactivate CDK2.

Minor concern:

1. Figure 3C: Mislabeled? Should the yellow bar represent the DMSO group? BTW, “7” in STM2457 is also missing.

Response： We appreciate your diligence in reviewing our manuscript. In this revision, Figure 3C has been updated to include new results from ^{13}C-labeled glucose tracing experiments, which provide a more detailed analysis of the metabolic shift towards the pentose phosphate pathway following METTL3 knockdown. We have also corrected the typographical error regarding the missing “7” in STM2457.

2. Results section and figure legends: All the titles are present in a very vague way. The reader can’t absorb any key information from the titles. The authors should change them to be more accurate and more conclusive.

Response: We have revised the titles in the results section and figure legends to be more specific and conclusive. Each title now more accurately reflects the experimental outcomes and the conclusions drawn from the data. Thank you for your valuable feedback, which has helped us to refine our manuscript and make it more accessible to our readers.

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