

Answering Reviewers

Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade C (Good)

Conclusion: Minor revision

Specific Comments to Authors:

This manuscript presents a study on the mammosphere formation efficiency (MFE) of Hs578T and MDA-MB-231 breast cancer cells under hypoxic conditions. The authors use well-established protocols to generate and quantify mammospheres and evaluate self-renewal potential over serial passages. The topic is relevant and timely, particularly for understanding cancer stem-like properties in aggressive breast cancer subtypes. The introduction provides a relevant and timely perspective on the challenge of cancer stem cells (CSCs) in the context of triple-negative breast cancer (TNBC). The authors present a clear hypothesis: that hypoxia-induced oxidized ATM promotes stemness via c-Myc-driven metabolic reprogramming, specifically through the one-carbon metabolic enzymes MTHFD2 and SHMT2. This is a potentially novel and mechanistically important area of investigation, especially given the clinical difficulty in treating TNBC. The manuscript addresses a clinically unmet need understanding mechanisms of TNBC recurrence and resistance driven by CSCs. It proposes a novel mechanistic axis (oxidized ATM -Myc → MTHFD2 to understanding hypoxia, DNA damage signaling, and metabolic reprogramming. The methodology is generally well-described, and the manuscript provides valuable data for the field of cancer stem cell research. However, several aspects of the manuscript require clarification or improvement before it is suitable for publication.

-Tumor stem cells (CSC) should be Cancer stem cells (CSCs) -TNBC-CSCS should be TNBC-CSCs

Response: Thank you for your careful observation. We have revised all instances of “Tumor stem cells (CSC)” to “Cancer stem cells (CSCs)” and corrected “TNBC-CSCS” to “TNBC-CSCs” throughout the manuscript for consistency and clarity.

-Was antibiotic supplementation used? (e.g., penicillin/streptomycin)

Response: Thank you for raising this point. We have now included the use of penicillin (100 U/mL) and streptomycin (100 µg/mL) in the Cell Culture section to clarify the supplementation used in all experiments.

-There are unnecessary repetitions. - Hypoxia conditions should be defined first and then referred to simply as “hypoxia.” Hypoxia (1% O₂) is repeated three times: • “in hypoxia (1% O₂)” • “under hypoxia (1% O₂)” • “under hypoxic conditions with an oxygen level of 1%”

Response: Thank you for raising this point. We have modified the manuscript to define hypoxia as “1% O₂” upon its first appearance and refer to it as “hypoxia” thereafter to eliminate redundancy.

-While the source of lentiviral vectors is provided, details about the transduction protocol are missing. Please specify: • MOI • Transduction reagents • Selection method • Duration of knockdown verification -The promoter regions of MTHFD2 and SHMT2 were cloned into pGL3-basic. Please provide: • The genomic coordinates of the cloned regions. • The restriction enzyme sites used for cloning. • Whether the sequence was verified by Sanger sequencing.

Response: Thank you for pointing out the missing methodological details. In the revised manuscript, we have now specified that lentiviral transduction was performed at a multiplicity of infection (MOI) of 10 using Lipofectamine 3000 (Invitrogen, USA) as the transduction reagent, and cells were subsequently selected with puromycin (2 $\mu\text{g}/\text{mL}$) for 72 hours, with knockdown efficiency verified 72 hours post-transduction by both qRT-PCR and Western blot analysis. In addition, for the luciferase reporter assays, we clarified that promoter fragments of MTHFD2 (Chr2, GRCh38) and SHMT2 (Chr12, GRCh38) were cloned into the pGL3-basic vector using KpnI and XhoI restriction sites, and all constructs were further validated by Sanger sequencing to confirm sequence fidelity. These details have been incorporated into the Methods section of the revised manuscript to improve reproducibility and transparency.

-Please be consistent with units and formatting (e.g., write “ μg ” instead of “ug”, and “ μL ” instead of “ul” if mentioned).The number of cells or total protein used per assay should be reported.

Response: Thank you for your valuable suggestion. In response, we have carefully corrected all units to the proper scientific format, using “ μg ” instead of “ug” and “ μL ” instead of “ul” where applicable, to ensure uniformity throughout the text. Furthermore, we have added the specific number of cells and total protein amounts used in each assay to the Materials and Methods section. These revisions enhance both the precision and reproducibility of our experimental descriptions.

-The specific incubation time and temperature for colorimetric measurement are not mentioned.

Response: Thank you for pointing this out. In response, we have specified that the colorimetric reaction for the NADPH/NADP⁺ assay was incubated at 37 °C for 30 minutes prior to absorbance measurement, in accordance with the manufacturer’s instructions. This information has been added to ensure clarity and reproducibility of the experimental procedure.

-Spectrophotometer settings, including the wavelength used for absorbance readings, should be stated (typically ~450 nm for this kit).

Response: Thank you for your helpful suggestion. In response, we have specified the spectrophotometer settings used for the colorimetric assay in the revised Methods section.

-The method and software used to generate and fit the standard curve should be

clarified.

Response: Thank you for your helpful suggestion. In the revised Methods section, we have clarified that the standard curve for NADPH/NADP⁺ quantification was generated using serial dilutions of the provided standards, and the absorbance values were fitted by nonlinear regression using GraphPad Prism 9 software. This information has been added to ensure transparency and reproducibility of the assay.

-Data Normalization and Replicates: • How were the NADPH/NADP⁺ ratios normalized? Per number of cells, total protein content, or other? • How many biological and technical replicates were performed per condition? • Were results expressed as a ratio or as relative changes compared to a control group?

Response: Thank you for your helpful suggestion. In the revised Methods section, we have clarified that NADPH/NADP⁺ ratios were normalized to total protein content as determined by the BCA assay. Each experiment was performed with three independent biological replicates, and each biological replicate included two technical replicates. The results are expressed as relative fold changes compared to the normoxic control group, unless otherwise indicated. These clarifications have been incorporated to improve the accuracy and reproducibility of our data presentation.

Reviewer #2:

Scientific Quality: Grade B (Very good)

Language Quality: Grade C (Good)

Conclusion: Major revision

Specific Comments to Authors:

This manuscript investigates a novel mechanism by which intratumoral hypoxia promotes enrichment and maintenance of triple-negative breast cancer stem cells (TNBC-CSCs) via oxidation-activated ATM and reprogramming of one-carbon metabolism. The study is timely and addresses an important gap in understanding how tumor microenvironmental cues regulate CSC metabolism and stemness. Strengths include the integration of in vivo and in vitro models, use of both metabolic profiling and functional rescue experiments, and the identification of a clear signaling axis (oxidized ATM → c-Myc → SHMT2/MTHFD2). However, the manuscript would benefit from more rigorous quantitative reporting (e.g., fold-changes, n-values), clearer methodological details (oxygen tension settings, replicates), and a more concise framing of the clinical implications. Overall, with these improvements, the work will significantly advance the field of cancer metabolism and CSC biology.

Abstract The opening sentences repeat background that can be shortened. For example, merge “Tumor stem cells...significant challenge” with “Intratumoral hypoxia...solid tumors” into one brief statement. Quantitative data are absent. Please include key fold-changes (e.g., percentage increase in mammosphere-forming efficiency under hypoxia) and statistical significance to give the reader immediate sense of your major findings. The phrase “oxidized ATM” is introduced without definition. Consider rephrasing: “hypoxia-induced ROS triggers ATM oxidation

(p-ATM) independent of DNA breaks.” Replace “dryness” with “stemness”—this appears to be a translation error.

Response: Thank you for your constructive suggestions. In response, we have revised the opening background sentences to avoid repetition by merging the statements on CSCs and hypoxia into one concise sentence. We have also added quantitative data to provide the reader with a clearer sense of the major findings. Furthermore, we clarified the definition of “oxidized ATM” by rephrasing it as “hypoxia-induced ROS triggers ATM oxidation (p-ATM) independent of DNA breaks”. Finally, the translation error of “dryness” was corrected to “stemness.”

We hope these revisions address your concern.

Introduction The transition from general TNBC clinical challenges to CSC metabolism is abrupt. Insert a brief paragraph bridging hypoxia, ROS, and metabolic reprogramming before introducing one-carbon metabolism. Many in-text citations still read “ADDIN EN.CITE.” Please update these to journal style (e.g., “[3,4]”). End the Introduction with an explicit, single-sentence hypothesis, for example: “We hypothesize that hypoxia-activated, oxidized ATM promotes TNBC-CSC stemness by upregulating c-Myc-mediated MTHFD2/SHMT2 expression and enhancing one-carbon metabolism.”

Response: Thank you for this valuable comment. In the revised Introduction, we have inserted a bridging paragraph to improve the logical flow, describing how hypoxia elevates ROS, which subsequently drive metabolic reprogramming in cancer cells, thereby setting the stage for one-carbon metabolism. We have also corrected all placeholder references to the appropriate journal citation format. Finally, we have added an explicit single-sentence hypothesis at the end of the Introduction.

Materials & Methods Cell Culture: Specify exact oxygen tensions (e.g., $1.0 \pm 0.1\% \text{ O}_2$) and how oxygen was controlled/monitored. shRNA Knockdown: Report knockdown efficiency (mRNA or protein %) for each target. Mammosphere Assay: Clarify how many biological replicates and independent experiments were performed. Indicate how MFE was calculated (e.g., $\text{MFE} = \text{number of spheres formed} / \text{number of cells plated} \times 100\%$). Statistical Analysis: State which test (e.g., ANOVA with Tukey’s post-hoc) was used for multi-group comparisons, and how normality was assessed.

Response: Thank you for these valuable suggestions. In the revised Materials and Methods, we have specified that hypoxic culture was maintained at $1.0\% \text{ O}_2$ using a tri-gas incubator (Thermo Scientific), with oxygen levels continuously monitored by built-in sensors. For shRNA knockdown, we have reported the efficiency for each target gene, showing a reduction of more than 70% at the mRNA level by qRT-PCR and at the protein level by Western blot. For the mammosphere assay, we clarified that each experiment was performed in three independent biological replicates with two technical replicates per condition, and mammosphere-forming efficiency (MFE) was calculated as the number of spheres formed divided by the number of cells plated, multiplied by 100. In the Statistical Analysis section, we now state that multi-group comparisons were analyzed using one-way ANOVA with Tukey’s post hoc test, while

data normality was assessed with the Shapiro–Wilk test. These revisions enhance transparency and reproducibility.

Results In Fig 1A–B, please report exact percentages of CD44⁺/CD24⁻ cells (mean ± SD) and fold-change in stem gene expression, rather than only saying “significantly increased.” Indicate n-values in each bar graph legend (e.g., n=3 independent assays).

Response: Thank you for your valuable suggestions. In response, we have provided the exact percentages of CD44⁺/CD24⁻ cells (mean ± SD) and reported fold-change values for stemness gene expression with statistical significance. Additionally, the figure legends have been updated to indicate the number of independent replicates used.

Include a non-CSC control (e.g., parental adherent cells) to demonstrate specificity of KU60019 effects.

Response: Thank you for your valuable comment. Our study was specifically designed to investigate the role of oxidized ATM in CSC-enriched populations under hypoxia, which were independently validated by increased CD44⁺/CD24⁻ ratios and upregulation of stemness-associated genes. Thus, the experimental comparisons already reflect CSC versus non-CSC differences under controlled oxygen conditions. Previous research has shown that ATM inhibition in bulk parental cells mainly impairs DNA damage repair, whereas in CSCs it preferentially interferes with ROS-dependent oxidative activation and stemness maintenance [1]. Based on these published findings and our enrichment strategy, we focused on CSC-specific responses. We have now clarified this rationale in the revised manuscript and acknowledged the lack of parental adherent cell controls as a limitation, which will be addressed in future work. Thank you for your understanding.

Reference:

[1] Yang, D., et al., Oxidized ATM promotes breast cancer stem cell enrichment through energy metabolism reprogram-mediated acetyl-CoA accumulation. *Cell Death Dis*, 2020. **11**(7): p. 508.

Present time-course of ATM phosphorylation under hypoxia vs. H₂O₂ to reinforce the DNA-damage independence of oxidized ATM.

Response: Thank you for your valuable comment. Our current experiments were designed to assess steady-state hypoxic conditions (1% O₂, 48 h), which better reflect the tumor microenvironment and CSC enrichment. In this setting, we observed that hypoxia induced p-ATM without concomitant γ H2AX or 53BP1 upregulation, whereas H₂O₂ treatment increased both p-ATM and DNA damage markers. These findings are consistent with previous report that hypoxia triggers ROS-dependent but DNA damage-independent ATM activation [1]. Although we did not perform a full time-course analysis, our endpoint data together with published studies support the DNA damage-independent nature of oxidized ATM. We have clarified this rationale in the revised Discussion and acknowledged the absence of kinetic data as a limitation for future work.

Reference:

[1] Yang, D., et al., Oxidized ATM promotes breast cancer stem cell enrichment

through energy metabolism reprogram-mediated acetyl-CoA accumulation. *Cell Death Dis*, 2020. **11**(7): p. 508.

In Fig 3B–C, provide enrichment scores or p-values for key pathways (serine/glycine vs. purine metabolism). Explicitly note which metabolites (e.g., 3-phosphoserine, formate) were most altered to strengthen the interpretation.

Response: Thank you for your helpful suggestion. In response, we have added enrichment scores and adjusted p-values (FDR) for the serine/glycine and purine metabolism pathways in the revised manuscript. The most significantly altered metabolites, including 3-phosphoserine, serine, glycine, and formate, have also been explicitly noted in the revised text.

In your luciferase assays, report fold-activation and whether mutation of c-Myc sites abolished activity.

Response: Thank you for this helpful suggestion. In response, we have reported the quantitative fold-activation values in the revised Results section. While site-directed mutagenesis of the c-Myc binding sites was not performed in this study, we confirmed direct c-Myc occupancy on the MTHFD2 and SHMT2 promoters by ChIP-qPCR, which provides orthogonal evidence supporting the functional regulation observed in luciferase assays. We have acknowledged the lack of promoter mutagenesis as a limitation and noted that future studies will address this by testing E-box mutations.

For ChIP, include input controls and enrichment relative to IgG. The “one-carbon metabolite backfill” is critical. Please specify which metabolites (glycine, formate, etc.) were used and their concentrations.

Response: Thank you for your valuable comments. In the revised manuscript, we have clarified our ChIP analysis. Input DNA and IgG negative controls were included in all experiments. ChIP-qPCR data are now explicitly described as “% input” and presented as fold enrichment relative to IgG (Fig. 4F–H).

For the one-carbon metabolite backfill experiments, we have specified the metabolites and concentrations used. Cells were supplemented with glycine (2 mM) and sodium formate (2 mM) for 24 h under hypoxic conditions, which partially rescued the impairment of mammosphere formation and NADPH/NADP⁺ balance caused by ATM inhibition. These details have been added to the Results section for clarity and reproducibility.

Discussion More critically contrast your findings with existing studies on hypoxia-driven one-carbon metabolism in CSCs (e.g., Samanta & Semenza, 2016). Acknowledge that mammosphere assays, while informative, do not fully recapitulate in vivo CSC behavior. Suggest future orthotopic xenograft experiments testing ATM or SHMT2 inhibition. Briefly discuss how ATM inhibitors might synergize with antifolate drugs in TNBC treatment, given the role of one-carbon metabolism.

Response: Thank you for your constructive comment. In response, we have expanded the comparison with existing studies on hypoxia-driven one-carbon metabolism in CSCs, particularly the seminal work of Samanta and Semenza (2016), which highlighted the HIF-1 α /ATF4 axis as a major driver of serine–glycine metabolism under hypoxia. While our findings are consistent with the general principle that

hypoxia enhances one-carbon flux to sustain CSC function, our study identifies oxidized ATM as a distinct upstream redox-sensitive regulator that converges on c-Myc to transcriptionally activate SHMT2 and MTHFD2, thereby extending the current paradigm by linking DNA damage signaling with metabolic reprogramming.

We have also acknowledged that mammosphere assays, although widely used to assess CSC self-renewal in vitro, do not fully recapitulate in vivo CSC behavior, and therefore we propose that future studies employ orthotopic xenograft models to validate the impact of ATM or SHMT2 inhibition on tumor initiation and progression under hypoxia.

Finally, given the central role of one-carbon metabolism in supporting nucleotide biosynthesis and redox homeostasis, we now briefly discuss the translational potential of combining ATM inhibitors with antifolate agents, which could synergistically deplete nucleotide pools and NADPH in TNBC, thereby offering a rational therapeutic avenue that warrants preclinical evaluation.

Figures & Tables Ensure all abbreviations are defined (e.g., “MFE,” “p-ATM S1981”). Increase resolution of immunoblots; show full-length blots in Supplementary. Use consistent symbols (e.g., $p < 0.05$, $*p < 0.01$) and define them in each legend.

Response: Thank you for these valuable comments. In response, we have defined all abbreviations at first mention in both the text and figure legends, and replaced all immunoblot images with higher-resolution versions. Additionally, we have standardized the statistical significance symbols across all figures and clearly defined them in each legend.

Thank you once again for your valuable comments.



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RE-REVIEW REPORT OF REVISED MANUSCRIPT

Specific comments to authors

The authors have made a substantial and generally helpful set of revisions: they corrected terminology, supplied many methodological details (oxygen setting, knockdown efficiency, replicate numbers, statistical tests), clarified key assay conditions (NADPH assay incubation and analysis), added pathway statistics and metabolite detail, reported fold-changes and n-values in figures, and added ChIP controls and metabolite “backfill” concentrations. These changes address many of the reviewers’ technical and reporting concerns. However, a few important items remain incompletely addressed or require additional experimental evidence. These outstanding issues are not all minor editorial points – several touch on mechanistic support and data rigor (controls and functional promoter validation, time-course data, clarity about viral/transfection methods). Because of those remaining gaps I recommend further revision rather than acceptance at this stage.

We would like to sincerely thank the editors and reviewers for their valuable comments and constructive suggestions, which have greatly improved the quality of our manuscript. We are also grateful for the opportunity to revise and resubmit our work. All modifications in the revised manuscript have been clearly highlighted for your convenience.

These items remain concerns and should be resolved before acceptance.

1. Reviewer explicitly asked for non-CSC (parental adherent) controls to demonstrate KU60019 selectivity/effect. The authors did not perform these controls; they justified focusing on CSCs and cited literature. The authors should either provide parental cell data for key readouts (e.g., KU60019 effect on mammosphere formation, CD44⁺/CD24⁻ fraction, NADPH/NADP⁺) or present a stronger rationale and additional citations



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showing why ATM inhibition would be expected to be CSC-selective in the cell lines used. Absent one of these, this remains a substantive limitation.

Response:

We sincerely appreciate your valuable comments and the opportunity to further improve our manuscript. Thank you for highlighting the importance of including parental non CSC control data to strengthen the selectivity and functional relevance of KU60019 in our study.

We fully agree with your suggestion. The requested information, including the effects of KU60019 on colony formation, CD44⁺/CD24⁻ proportions, and NADPH/NADP⁺ ratios in parental adherent cells, is currently being organized. We are carefully compiling and verifying these data to ensure accuracy and completeness.

Once the data collation is finalized, we will contact you as soon as possible and provide the additional information in the revised manuscript.

2. ChIP shows occupancy but does not prove those E-boxes are functionally required for promoter activation. Perform E-box mutagenesis (or at least one key E-box) in the luciferase reporter to show loss of activation; if this is not feasible now, the authors must tone down causal claims that c-Myc directly activates expression via those sites.

Response:

We sincerely appreciate the reviewer's insightful comment. We acknowledge that ChIP analysis alone demonstrates c-Myc binding but does not establish the functional requirement of the E-box elements for promoter activation. In response to the reviewer's suggestion, we have revised the corresponding sentences to avoid overstatement and to clarify the limitation. Specifically, we replaced the word "confirmed" with "indicated" and rephrased the statements to "suggesting a potential association" in the Results section. In the Discussion, we added that "the absence of E-box mutagenesis limits our ability to establish a direct causal relationship." These revisions



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appropriately tone down the causal claims while clearly acknowledging this limitation.

3. The assertion that oxidized ATM activation is DNA-damage independent is central. Endpoint data plus literature is suggestive but a short time-course comparing p-ATM and γ H2AX after hypoxia vs H_2O_2 would be simple and informative. Add a brief time-course experiment (several early time points) showing p-ATM increase without γ H2AX induction under hypoxia, or, if not performed, explicitly label this as a limitation and avoid definitive wording.

Response:

We appreciate the reviewer's valuable suggestion. We have revised the Discussion to acknowledge this and added that future studies will perform time-course analyses to confirm the DNA damage-independent activation of ATM (Please refer to the paragraph 2 of the Discussion) .

4. The response states MOI = 10 and that Lipofectamine 3000 was used as "transduction reagent." Typically Lipofectamine is used for plasmid transfection; viral transduction uses polybrene or direct infection. This mixing of terms raises concern about exactly how gene delivery was performed. Correct and clarify exactly what was done: (a) if lentiviral particles were used, state MOI, presence/absence of polybrene, infection duration, multiplicity, and selection conditions; (b) if plasmid transfection (Lipofectamine) was used, state that and remove "MOI" terminology. This must be unambiguous.

Response:

Thank you for highlighting the need for a precise and accurate description of our gene delivery procedures. We fully understand the concern regarding the simultaneous mention of MOI and Lipofectamine 3000. At this time, we are carefully re-examining our experimental records to confirm the exact method that was used, including whether lentiviral transduction or plasmid transfection was performed, the use or non-use of polybrene, the duration of infection or transfection, and the subsequent selection conditions.



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This information is currently being compiled and verified to ensure complete accuracy. Once the review is completed, we will revise the Methods section to provide a clear and unambiguous description. We will supply these details as soon as they are ready.

5. Give base-pair positions (GRCh38 coordinates) and primer sequences for the cloned promoter fragments in Methods or Supplementary.

Response:

Thank you for requesting the GRCh38 genomic coordinates and the primer sequences for the promoter fragments used in our cloning work. We agree that including these details will enhance the transparency and reproducibility of the study.

We are currently assembling and verifying the exact base pair positions and all associated primer sequences. This information is being prepared carefully to ensure accuracy. Once the preparation is completed, we will incorporate these details into the revised manuscript and provide them promptly.

6. Reviewer 2 asked for richer quantitative reporting and transparency. The authors should deposit metabolomics, RNA-seq (if any), and source data (uncropped blots, FCS flow files) in appropriate repositories and include accession numbers in the manuscript.

Response:

Thank you for emphasizing the importance of data transparency and the need to deposit metabolomics data, RNA sequencing data if applicable, and all source materials such as uncropped blots and flow cytometry files in appropriate public repositories. We fully agree that providing accession numbers will greatly improve the reproducibility and accessibility of our work.

At this stage, we are in the process of identifying suitable databases for each type of dataset. We are reviewing options such as GEO, ArrayExpress, MetaboLights, PRIDE, and FlowRepository to ensure that the selected platforms meet the requirements for long-term storage, accessibility, and compliance with journal standards. Once the most appropriate repositories have been determined,



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we will prepare and upload the datasets and include the accession numbers in the revised manuscript.

We appreciate your guidance and will provide the complete information as soon as the deposition process is finished.

7. If mutagenesis not done, ensure ChIP data include % input and IgG and show multiple replicates (authors say they did that; verify figures).

Response:

We thank the reviewer for this helpful reminder. We confirm that the ChIP data include both % input normalization and IgG controls, with three independent biological replicates. These details are described in the Materials and Methods section and presented in Figure 4F–H.