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Peer Review File

Hydrogen Sulfide Coordinates Glucose Metabolism Switch through Destabilizing Tetrameric Pyruvate Kinase M2

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the manuscript titled "Hydrogen sulfide coordinates glucose metabolism switch through destabilizing tetrameric pyruvate kinase M2" by Rong-Hsuan Wang, et al., the authors put forth a hypothesis that H2S production primarily by the enzyme CBS leads to the persulfidation of PKM2 at cysteine 326, which drives the destabilization of its enzymatically active tetrameric form driving metabolism away from OXPHOS to a more glycolytic state and enabling the dimer form of PKM2 to enter the nucleus and aid in the transcription of tumor-supportive genes.

The authors perform a number of cellular, biochemical, and biophysical experiments highlighted by the PKM2C326S mutant that is not able to be persulfidated at the cysteine 326 site. This mutant form bolsters the findings of the authors and leads to a plausible mechanism for increase breast cancer cell growth in culture and in vivo. These findings will enable a more targeted approach for treatment of H2S-supported tumors by focusing on PKM2 and the metabolic shifts persulfidation of PKM2 results in.

However, I have listed below several comments, suggestions, and questions to be addressed by the authors in order to clarify some questions I have with the manuscript as well as improve the scientific rigor and clarity of this study.

1) Lines 127 and 128: It would be helpful to state here what type of cancer cell types are used in the paper.

2) Line 166: Aminooxyacetic acid (AOAA) is not just an H2S inhibitor. It is a general inhibitor of enzymes that utilize vitamin B6 (aka PLP). As both CBS and CTH utilize vitamin B6, it does inhibit their activity to produce H2S, but will also have many other targets that it inhibits that are not related to H2S generation. They authors should use more specific inhibitors (such as PAG to inhibit CTH) and/or knockdown of CBS or CGL to display their point related to nuclear translocation and cell proliferation of MDA-MB-231 cells. Likewise, throughout the manuscript when AOAA is utilized, these experiments should be backed up with genetic knockdown or knockout of CBS and/or CTH.
See: Mei-Yu Geng, Hiroshi Saito, Hiroshi Katsuki,
Effects of vitamin B6 and its related compounds on survival of cultured brain neurons,
Neuroscience Research,
Volume 24, Issue 1,
1995,
Pages 61-65,
ISSN 0168-0102,
https://doi.org/10.1016/0168-0102(96)81279-X.
(https://www.sciencedirect.com/science/article/pii/016801029681279X)

3) Starting at line 304: How do the authors resolve the somewhat contrary findings of having the level of lactate increased in cells expressing PKM2C326S compared to that in wildtype PKM2-expressing cells, but then stating a few lines down that blocking PKM2 sulfhydration at C326 facilitates mitochondrial OXPHOS by increasing PK activity? Wouldn't it be suspected that if OXPHOS is enhanced that there would be a decreased level of lactate?

4) Line 544: Why were the cells switched for a serum-free medium when adding the NaHS, but kept in growth medium wen adding AOAA? Do you think that by removing serum you may also be impacting cellular growth that is not properly controlled for when keeping it in the media with serum when adding AOAA? Likewise, it was previously published that serum removal acts as a strong inducer of endogenous H2S production (see Jiang X, MacArthur MR, Treviño-Villarreal JH, Kip P, Ozaki CK, Mitchell SJ, Mitchell JR. Intracellular H2S production is an autophagy-dependent adaptive response to DNA damage. Cell Chem Biol. 2021 Dec 16;28(12):1669-1678.e5. doi: 10.1016/j.chembiol.2021.05.016. Epub 2021 Jun 23. PMID: 34166610; PMCID: PMC8665944.)

5) Line 563: It is not clear how the biotin thiol assay could have been successful if the authors added 1mM DTT prior to the addition of biotin-HPDP (and not after). In the author's scenario, any persulfide formed either endogenously or through the addition of NaHS would be removed by this

DTT treatment, and thus return the cysteine residues to their normal -SH thiol state. Thus, there would be no differences detected using their method, yet they report data in which there are differences detected plus/minus NaHS.

6) In the methods in general, how was the persulfide modification detected by mass spec? Usually, this requires pull down/isolation of persulfidated proteins with a final reducing step utilizing DTT or TCEP, and then followed with utilization of isobaric labeling of the previously persulfidated peptide with a reagent such as iodoTMT. I have a hard time understanding how the authors simply subjected proteins into the mass spec and it was sufficient to identify the persulfide modification on PKM2.

7) To ensure that AOAA isn't simply killing the cells resulting in the proliferation data presented in Figure 1F, the authors should perform a rescue experiment where AOAA and NaHS treatment are combined on the same culture. This would also help define that it is only the lack of H2S produced from AOAA treatment and not the inhibition of the possible 100's of enzymes utilizing vitamin b6 that causes its lack of proliferation.

8) The authors have not empirically shown that AOAA treatment on their cells at the dose chosen inhibits H2S production and/or levels. The authors should perform these experiments.

9) While it is appreciated the authors have identified a possible therapeutic target for the treatment of the specific cancer types tested in the article (in which H2S and persulfidation support cancer growth), they should also discuss more the cancer types in which H2S acts as a tumor suppressor (see Hellmich MR, Szabo C. Hydrogen Sulfide and Cancer. Handb Exp Pharmacol. 2015;230:233-41. doi: 10.1007/978-3-319-18144-8_12. PMID: 26162838; PMCID: PMC4665975 and Zhang, Y., Chen, S., Zhu, J. et al. Overexpression of CBS/H2S inhibits proliferation and metastasis of colon cancer cells through downregulation of CD44. Cancer Cell Int 22, 85 (2022). https://doi.org/10.1186/s12935-022-02512-2)

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors intend to explore the potential mechanism of reducing PKM2 enzyme activity in cancer cells, which allows the cells to attain a more significant fraction of glycolytic metabolites needed for rapid cell proliferation. They first show that hydrogen sulfide (H2S) destabilizes PKM2 tetramer into dimer/monomer, leading to reduced PKM2 enzyme activity and an increase in the activation of nuclear transcriptional genes mediated by dimeric PKM2. In addition, Proteomic profiling of endogenous PKM2 reveals the occurrence of sulfhydration at cysteines, notably at cysteine 326. Blocking PKM2 sulfhydration at cysteine 326 through amino acid mutation stabilizes PKM2 tetramer and crystal structure, further revealing PKM2C326S is a newly identified form. The presence of a PKM2C326S mutant in cancer cells effectively rewires glucose metabolism to mitochondrial respiration, significantly inhibiting tumor growth.

Cancer cells reprogram their glucose metabolic pathway from oxidative phosphorylation toward aerobic glycolysis. Pyruvate kinase M2 (PKM2), which converts phosphoenolpyruvate (PEP) to pyruvate, is the rate-limiting enzyme in cancer glucose metabolism. However, the mechanism underlying the decreased PKM2 activity in cancer cells remains elusive. In this regard, this study could have potential scientific and clinical significance. The other significant strengths of this manuscript include the novelty of identification of the H2S effect on sulfhydration at PKM2 cysteine 326 position and the solid biochemical and protein structure analysis employed in this study.

However, several weaknesses were identified in this manuscript. The most significant drawback is that the whole manuscript depends on one breast cancer cell line, MDA-MB231. No confirmation experiments were performed in human tissues, which significantly limit the impact of the current

study. Other weaknesses include the lack of normal control and animal numbers in mouse work, which further diminished the reviewer's enthusiasm for this study. These weaknesses are listed and discussed in detail as follows:

1) In Figure 1, two cell lines were mentioned in the text. Only the MDA231 cell line was used for all the experiments. More cancer cell lines should be included in this study to make the concept more general.

2) Does the NaHS treatment only impact cancer cell lines? What about the effect of NaHS on normal or immortalized cell models?

3) In Figures 1G-I, only MDA231 cells were used. This is not strong enough to conclude that cancer cells have higher SSH-PKM2 than normal cells. More cell lines are required.

4) In Figure 4A, the expression level of wt_PKM2 and PKM2-C326S mutant is not equal. The Mutant form showed more than 5-fold more expression than the WT form. This could impact the interpretation of the observed results in the following experiments. The same problem was shown in Figure S5, in which the WT form is expressed much higher than the mutant form.

5) In Figure 4A, the SSH-PKM2 level was not shown in the all the cell models.

6) It is hard to believe there is any difference between the lactate in Figure 4H.

7) I have a question about the ectopic expression model. We agree that the MDA231 is a cancer cell line that already expresses high SSH-PKM2 due to the H2S synthesizing enzyme CBS and CTH. Why this cell model was used to overexpress PKM? Why not choose a cell line with low SSH PKM2? I suspect the original SSH-PKM in the cells will impact the function of the expressed form.

8) In Figure 6, the mouse number in this study was shown between 3-10, which means the mouse number is only 3 in some groups. I am questioning how the statistical analysis was conducted.

9) It would be nice to show all the animal pictures instead of just 2 -3.

10) The tumor growth experiment in Figure 6B showed that MDA231 cells expressing C326S mutation did not show any tumor growth, which contradicts the in vitro cell growth results in Figure 6A. This phenomenon or effect deserves further discussion.

11) It was not discussed why the ectopic expression of wt-PKM2 did not affect metabolism, cell cycle, or cancer cell growth in vitro and in vivo in Figures 4, 5, and 6.

Reviewer #3 (Remarks to the Author):

Wang et al provide interesting evidence supporting the notion that sulfhydration of C326 in the M2 isoform of pyruvate kinase destabilizes the active tetrameric state of the enzyme and provides a mechanism of in vivo regulation of this key glycolytic enzyme. In this way, this PTM influences primary glucose metabolism and can promote tumorigenesis. The authors also present evidence that blocking the sulfhydration PTM at C326 through a C326S point mutation, leads to a more stable tetrameric enzyme assembly that in vivo inhibits tumour growth through alterations in glucose metabolism through stable PK activity that is insensitive to sulfhydration effects. The area of sulfhydration as a mechanism of regulatory post-translational modification (PTM) is an interesting and understudied area and the authors do a good job of presenting a comprehensive body of impactful work that suggests that modification of C326 may be an important in vivo regulatory mechanism of PK that could impact cancer tumorogenesis.

While the general conclusions are largely supported by the data I do have some comments that I feel should be addressed by the authors as they relate to the interpretation and impact of specific results.

1) In general, I don't find that the evidence provided fully supports the notion that sulfhydration results in a shift in the dimer-tetramer equilibrium. In my view, the substitution leads to a more

heterogeneous and dynamic population of states as the profiles are quite broad rather than a simple, stochastic shift in a population of states between the dimer and the tetramer. Further, the sizing data show that the WT in the absence of FBP populates the monomer/dimer state more than the treated enzyme in the presence of FBP begging the question of where is the data on the treatment of the enzyme in the absence of FBP? Perhaps I missed this data in my review but it would seem important to include. The authors state sulfhydration modulates the dissociation of PKM2 tetramers into dimers/monomers. Is it dimers or monomers? This distinction seems like an important one however as stated above, I don't feel the data supports the notion that a transition from one quaternary state to another is warranted as the data in my view suggest that the modification destabilizes the tetramer into a more complex/heterogeneous population of various oligomeric states and not a stochastic shift as the authors statements suggest. Further, the authors analyze the C326S mutant to state that it stabilizes the tetramer. Again, I don't think that this stochastic interpretation is supported by the data. Also, there appears to be an aggregate (large than tetramer) peak in the mutant that is ignored suggesting that a shift in the propensity to higher order states is also occurring. In general, I think a more thoughtful examination of the true effects of C326 modification and serine substitution and their influence on the nature of the enzymes quaternary state is warranted to be more consistent with the data that is provided.

2) The lack of recovery of full enzyme activity after DTT treatment (Fig 1B) needs to be explained by the authors. What is the general effect of NaSH treatment on the kinetic assay? The fact that WT activity is not fully recovered suggests that NaSH is causing other effects that reduce enzyme function or are irreversible or affecting other aspects/enzymes in the kinetic assay.

3) While perhaps not physiologically relevant, from a mechanistic standpoint it would be important to understand if the disruption in the quaternary state and the correlated activity changes by sulfhydration of C326 are specific to this modification or whether other chemical modifications of this residue result in similar effects. Related to this point, is modification to the sulfhydrated form necessary or can the general oxidation of this residue to sulfenic, sulfinic and sulfonic states accomplish the same functional effect? Lastly, I don't understand the statement that AOAA treatment supports the hypothesis that the effects at cysteine 326 are predominantly mediated through sulfhydration and not other modifications/general oxidation. Clarification of this point would be helpful.

4) It is unclear what the importance of understanding structural basis for the C326S mutation on tetramer stabilization is to the presented work. The mechanistic basis by which the serine substitution affects the oligomeric state of the enzyme seems tangential to the rest of the work as it provides no insight into the real question that is not answered and that is how the modification of C326 via sulfhydration stimulates the opposite effect. It is also hard to rationalize how the single substitution is supporting an alternate tetrameric form.

On a more basic level, I don't understand the interpretation where the authors state that replacing the Cys with Ser at residue 326 of PKM2 remotely changes the dynamics of the C domain and promotes tetramerization (Fig. 3). If I am interpreting the structures correctly, the R and T structures compared to this new structure have ligands bound to the C domain where this structure is unligated. If this is the case, how do the authors distinguish between differences that reflect influences of the mutation versus the more likely conclusion that the differences in the disorder/order of this domain are the result of the impact of comparing ligated to unligated states of the enzyme and the order in the domain that is correlated with ligand/allosteric effector binding.

Lastly, meaningful assessment of the interpretation of the structural data is impossible without access to the model and map files. Further to this point, the PDB deposition report was not provided as a supplemental file.

Minor Points

Several instances there is the statement the C326 can be a therapeutic target for anti-cancer drug development. I fail to see how a specific amino acid can be targeted for therapeutic development beyond gene therapy. The authors need to provide more context to what approach they are referring to or remove these unsupported statements.

The notion that pyruvate kinase (PK) regulates the final rate-limiting step of glycolysis by catalyzing the phosphoryl transfer from phosphoenolpyruvate (PEP) to adenosine diphosphate

(ADP) to produce pyruvate and ATP is not universally supported. While this is a thermodynamically irreversible step in the process it is not necessarily the solely rate limiting process as reported most recently by Zuo J, Tang J, Lu M, Zhou Z, Li Y, Tian H, Liu E, Gao B, Liu T, Shao P. Glycolysis Rate-Limiting Enzymes: Novel Potential Regulators of Rheumatoid Arthritis Pathogenesis. Front Immunol. 2021

On p.7 the authors state that FBP may dissociate into fructose by NaHS treatment. Are the authors intending to state that FBP is hydrolyzed into fructose and 2xPi? This statement should be clarified so it makes sense from a chemical standpoint.

The authors state that the protein concentration was determined via absorbance at 280nm however they do not provide an extinction coefficient for that calculation.

The crystallographic data table is incomplete. For instance, it lacks statistics and validation parameters such as Molprobity and Ramachandran analysis. Further, some sections make no sense such as 14857 under the heading of macromolecules. I assume the authors mean there were 14857 atoms in the macromolecular portion of the structural model. Average B factors should be broken down for the different components of the model such as solvent, macromolecules, heteroatoms as is normal convention not just an overall average B factor reported.

What do S and X mean in Table S1? These terms should be described in the table footnote.

There are some general English language/grammar issues throughout the manuscript in particular there is a notable lack of articles in many sentences. Careful editing of the work for these issues is recommended.

We thank the reviewers for dedicating their valuable time to review our work and providing insightful suggestions to enhance the manuscript's rigor and impact on cancer metabolism. In this revised version, we have incorporated the changes highlighted in red (please refer to the second file of the article).

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Response: Thank you. We have added this information to the paper (Line 122-132).

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general inhibitor of enzymes that utilize vitamin B6 (aka PLP). As both CBS and CTH utilize vitamin B6, it does inhibit their activity to produce H2S, but will also have many other targets that it inhibits that are not related to H2S generation. They authors should use more specific inhibitors (such as PAG to inhibit CTH) and/or knockdown of CBS or CGL to display their point related to nuclear translocation and cell proliferation of MDA-MB-231 cells. Likewise, throughout the manuscript when AOAA is utilized, these experiments should be backed up with genetic knockdown or knockout of CBS and/or CTH. See:
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1995,
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ISSN 0168-0102,
https://doi.org/10.1016/0168-0102(96)81279-X.
(https://www.sciencedirect.com/science/article/pii/016801029681279X)

Response: Thank you for the valuable suggestion. We understand AOAA is not specific H_2S inhibitors, but at present, there are no suitable inhibitors available to suppress CBS enzymatic activities specifically. To address this limitation, we have incorporated additional data involving both CBS and CTH knockdown by siRNA (Fig. S1F) to assess its effect on reduced PKM2 sulfhydration (Fig. S1G), NaHS-induced PKM2 nuclear translocation (Fig. S1J), NaHS-induced gene expression (Fig. S1K), the percentage of polyploid cells (>4N) (Fig. S7G), and the rate of cell proliferation (Fig. 1F).

3) Starting at line 304: How do the authors resolve the somewhat contrary findings of having the level of lactate increased in cells expressing PKM2C326S compared to that in wildtype PKM2-expressing cells, but then stating a few lines down that blocking PKM2 sulfhydration at C326 facilitates mitochondrial OXPHOS by increasing PK activity? Wouldn't it be suspected that if OXPHOS is enhanced that there would be a decreased level of lactate?

Response: Thank you for your questions. The statistical significance of the increase in lactate levels in cells expressing PKM2 C326S, as indicated in Figure 4H, is derived from six data points. These data points reflect consistent

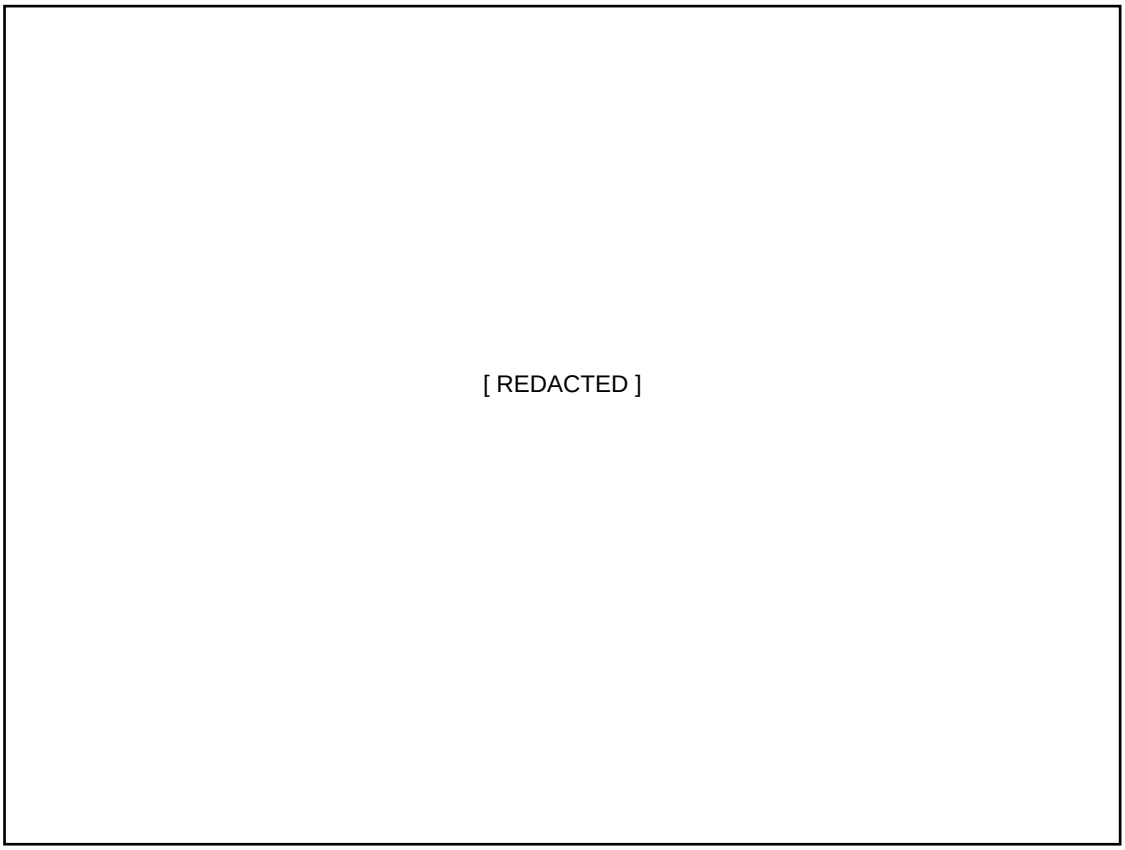
measurements with low variability, which provides the statistical power to detect even minor differences as significant. Given relatively comparable lactate levels between WT and C326S, it is possible that cells maintain a certain level of glycolysis to support biosynthetic pathways and redox balance while simultaneously utilizing pyruvate in the mitochondria more efficiently, hence the enhanced OXPHOS. Therefore, we have amended the sentence in the manuscript considering that the lactate level between wt-PKM2 and PKM2-C326S is similar (Line 334-336). Furthermore, we have extensively compared our results and addressed the paradoxical findings in the Discussion section (Line 526-548).

4) Line 544: Why were the cells switched for a serum-free medium when adding the NaHS, but kept in growth medium wen adding AOAA? Do you think that by removing serum you may also be impacting cellular growth that is not properly controlled for when keeping it in the media with serum when adding AOAA? Likewise, it was previously published that serum removal acts as a strong inducer of endogenous H2S production (see Jiang X, MacArthur MR, Treviño-Villarreal JH, Kip P, Ozaki CK, Mitchell SJ, Mitchell JR. Intracellular H2S production is an autophagy-dependent adaptive response to DNA damage. Cell Chem Biol. 2021 Dec 16;28(12):1669-1678.e5. doi: 10.1016/j.chembiol.2021.05.016. Epub 2021 Jun 23. PMID: 34166610; PMCID: PMC8665944.)

Response: Thank you for your valuable comments. To induce gene expression by different stimulants, such as EGF or GF, we employed serum-starvation medium prior to stimulation to reduce basal activities of cells, synchronized most cells at G0/G1 phase, and minimized the unwanted interference from the mixed components of serum (PMID: 21613612). Multiple studies have shown that FBS contains many unidentified molecules that could potentially affect the results of cell experiments (PMID: 36732616; 17022666). Hence, when we added NaHS, the H_2S donor, to the medium, we performed it in serum-free conditions. Conversely, we believe that conducting inhibition experiments under normal culture conditions would be more appropriate.

To address the comments that serum removal might influence cellular growth and induce H_2S production endogenously, as noted in the literature mentioned by the reviewer, we conducted a comparative analysis of gene expression under conditions with or without serum. As shown in the following figure, we observed that under serum-free conditions, the basal expression of

genes related to glycolysis, including LDHA, GLUT1, and GLUT12, was increased. The expression of other genes remains similar between conditions with or without serum. However, expressions of these genes were less affected by NaHS in the medium containing 10% FBS. Considering the high levels of protein-glutathione mixed disulfides found in FBS (PMID: 67079), it is possible that NaHS could interact with these compounds. As a result, gene induction by NaHS would be less pronounced in medium containing 10% FBS compared with that in serum-free medium. In light of this, we adjusted our stimulation protocols under serum-free conditions. In summary, our control groups were appropriately matched, whether under serum conditions or not. Therefore, even in serum-free conditions, the +NaHS group is compared to the control group, which is also serum-free.



5) Line 563: It is not clear how the biotin thiol assay could have been successful if the authors added 1mM DTT prior to the addition of biotin-HPDP (and not after). In the author's scenario, any persulfide formed either endogenously or through the addition of NaHS would be removed by this DTT treatment, and thus return the cysteine residues to their normal -SH thiol

state. Thus, there would be no differences detected using their method, yet they report data in which there are differences detected plus/minus NaHS.

Response: We only added DTT in certain conditions in Figs. 1A and S1A experiment to prove that the pulldown proteins are SSH-bond. We have modified the description of methods to clarify how we performed those experiment (Line 671-673).

6) In the methods in general, how was the persulfide modification detected by mass spec? Usually, this requires pull down/isolation of persulfidated proteins with a final reducing step utilizing DTT or TCEP, and then followed with utilization of isobaric labeling of the previously persulfidated peptide with a reagent such as iodoTMT. I have a hard time understanding how the authors simply subjected proteins into the mass spec and it was sufficient to identify the persulfide modification on PKM2.

Response: To specifically detect persulfide modifications on PKM2, we used methyl methanethiosulfonate (MMTS) to block all free thiol groups (convert free -SH groups into -SCH3), preventing oxidation in the solution. Subsequently, we conducted immunoprecipitation experiments using PKM2 antibodies to selectively enrich for PKM2. The immunoprecipitated PKM2 was then treated with iodoacetamide (IAM) to modify Cys-SSH residues, resulting in the formation of Cys-S-S-CAM. The modified samples were then subjected to Mass Spectrometry (MS) analysis. This targeted approach allowed us to focus on PKM2 persulfidation, which increased the specificity of our detection for persulfidation on PKM2, distinguishing it from PKM1. Please refer to the result section in our manuscript (lines 224-236), and for the representation of the process, please see Figure 2C.

7) To ensure that AOAA isn't simply killing the cells resulting in the proliferation data presented in Figure 1F, the authors should perform a rescue experiment where AOAA and NaHS treatment are combined on the same culture. This would also help define that it is only the lack of H2S produced from AOAA treatment and not the inhibition of the possible 100's of enzymes utilizing vitamin b6 that causes its lack of proliferation.

Response: We appreciate this suggestion of a rescue experiment to evaluate

the specific effects of AOAA on cell proliferation. The rescue experiment indicated that NaHS was only able to partially reverse the AOAA-induced reduction in cell proliferation (Data not shown). To further clarify the specific role of H_2S, we replaced the initial AOAA data with results from cells using CBS and CTH knockdown (Fig. 1F), revealing that the reduction in proliferation is due to decreased H_2S synthesis and not the broad inhibition of vitamin B6-dependent enzymes.

8) The authors have not empirically shown that AOAA treatment on their cells at the dose chosen inhibits H2S production and/or levels. The authors should perform these experiments.

Response: In response to this comment, we investigated the potential of AOAA treatment to inhibit H_2S production by lead acetate paper assays. Our data indicated that treatment with 0.25mM AOAA effectively inhibits the endogenous level of H_2S (Fig. S1E) and the formation of SSH-PKM2 (Fig. S1G). Furthermore, AOAA was observed to attenuate hypoxia-induced SSH-PKM2 in both MDA-MB-231 cells (Fig. 1I) and PC3 cells (Fig. S2H).

9) While it is appreciated the authors have identified a possible therapeutic target for the treatment of the specific cancer types tested in the article (in which H2S and persulfidation support cancer growth), they should also discuss more the cancer types in which H2S acts as a tumor suppressor (see Hellmich MR, Szabo C. Hydrogen Sulfide and Cancer. Handb Exp Pharmacol. 2015;230:233-41. doi: . PMID: 26162838; PMCID: PMC4665975 and Zhang, Y., Chen, S., Zhu, J. et al. Overexpression of CBS/H2S inhibits proliferation and metastasis of colon cancer cells through downregulation of CD44. Cancer Cell Int 22, 85 (2022). https://doi.org/10.1186/s12935-022-02512-2)

Response: Thank you. We have expanded our discussion to address the dual role of H_2S in cancer biology, including the studies mentioned by the reviewer (Lines 113-119). This balanced view helps to discuss the complexity of H_2S's role in cancer and supports the need for targeted strategies in the context of the specific cancer type and its unique metabolic environment.

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In this manuscript, the authors intend to explore the potential mechanism of

reducing PKM2 enzyme activity in cancer cells, which allows the cells to attain a more significant fraction of glycolytic metabolites needed for rapid cell proliferation. They first show that hydrogen sulfide (H2S) destabilizes PKM2 tetramer into dimer/monomer, leading to reduced PKM2 enzyme activity and an increase in the activation of nuclear transcriptional genes mediated by dimeric PKM2. In addition, Proteomic profiling of endogenous PKM2 reveals the occurrence of sulfhydration at cysteines, notably at cysteine 326. Blocking PKM2 sulfhydration at cysteine 326 through amino acid mutation stabilizes PKM2 tetramer and crystal structure, further revealing PKM2C326S is a newly identified form. The presence of a PKM2C326S mutant in cancer cells effectively rewires glucose metabolism to mitochondrial respiration, significantly inhibiting tumor growth.

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However, several weaknesses were identified in this manuscript. The most significant drawback is that the whole manuscript depends on one breast cancer cell line, MDA-MB231. No confirmation experiments were performed in human tissues, which significantly limit the impact of the current study. Other weaknesses include the lack of normal control and animal numbers in mouse work, which further diminished the reviewer's enthusiasm for this study. These weaknesses are listed and discussed in detail as follows:

1) In Figure 1, two cell lines were mentioned in the text. Only the MDA231 cell line was used for all the experiments. More cancer cell lines should be included in this study to make the concept more general.

Response: We acknowledge the reviewer's concern regarding the generalizability of our findings across different cancer cell lines. To address this concern, we expanded our study to PC3 cells, including the evaluation of

NaHS-suppressed PK activity (Fig. S1B), NaHS-induced nuclear translocation of PKM2 (Fig. S2E), NaHS-mediated transcriptional regulation (Fig. S2G), and hypoxia-induced PKM2 sulfhydration (Fig. S2H). These results not only confirm our observations in MDA-MB-231 cells but also demonstrate that the effects of NaHS treatment are consistent across various cancer cell types, suggesting broader applicability.

2) Does the NaHS treatment only impact cancer cell lines? What about the effect of NaHS on normal or immortalized cell models?

Response: In response to comment concerning the impact of NaHS on normal cells, we conducted additional experiments using MCF-10A cells, an immortalized human breast epithelial cell line. Our findings indicate the expression levels of H_2S-producing enzymes, CBS and CTH, are significantly lower in MCF-10A cells than other breast cancer cells (Fig. 1G). Correspondingly, there were lower SSH-PKM2 levels (Fig. 1H). Meanwhile, in the presence of NaHS, we observed stimulation of nuclear translocation of PKM2 (Fig. S2D), leading to enhanced expressions of PKM2-mediated genes (Fig. S2F). These effects mirror those observed in MDA-MB-231 and PC3 cells, indicating a potentially universal mechanism of NaHS in influencing PKM2 behavior, despite a differential baseline expression of H_2S-producing enzymes in the immortalized normal cells.

3) In Figures 1G-I, only MDA231 cells were used. This is not strong enough to conclude that cancer cells have higher SSH-PKM2 than normal cells. More cell lines are required.

Response: To address this issue, we investigated two additional breast cancer cell lines, MCF-7 and HCC-1395. Intriguingly, our findings revealed a significantly higher expression of CBS in three cancer cell lines, while MDA-MB-231 and HCC-1395 exhibited elevated levels of CTH (Fig. 1G). Of particular note, the level of SSH-PKM2 was markedly increased in all three cancer cell lines compared to MCF-10A (Fig. 1H). These results across multiple cell lines substantiate the hypothesis that the upregulation of H_2S levels by elevated expression of CBS or CTH in breast cancer cells leads to the enhanced sulfhydration of PKM2.

4) In Figure 4A, the expression level of wt_PKM2 and PKM2-C326S mutant is

not equal. The Mutant form showed more than 5-fold more expression than the WT form. This could impact the interpretation of the observed results in the following experiments. The same problem was shown in Figure S5, in which the WT form is expressed much higher than the mutant form.

Response: We have thoroughly assessed the expression levels of overexpressed PKM2 and have quantified the expression levels of the PKM2-WT and the PKM2-C326S mutant (Fig. 4A, S6A). The mutant form exhibited approximately a 2-fold increase in V5-overexpression compared to the WT form in both MDA-MB-231 and PC3 cells. Furthermore, we observed the expression of the V5-tagged PKM2 WT form decreased over time, while the C326S form remained unaffected. This observation implies the presence of a negative feedback mechanism associated with PKM2 overexpression. This mechanism could involve the higher degree of NaHS-induced nuclear translocation of PKM2-WT (Fig. 4I), potentially leading to epigenetic changes that suppress its own expression. More investigation will be needed to clarify this possibility.

5) In Figure 4A, the SSH-PKM2 level was not shown in the all the cell models.

Response: To address this, we performed the Biotin switch assays to detect the persulfidated form of PKM2 in the stable cell lines. The Western blot and quantitative analysis are shown in Figure 4A.

6) It is hard to believe there is any difference between the lactate in Figure 4H.

Response: The statistical significance of the increase in lactate levels in cells expressing PKM2 C326S, as indicated in Figure 4H, is derived from six data points. These data points reflect consistent measurements with low variability, which provides the statistical power to detect even minor differences as significant. Given relatively comparable lactate levels between WT and C326S, it is possible cells maintain a certain level of glycolysis to support biosynthetic pathways and redox balance while simultaneously utilizing pyruvate in the mitochondria more efficiently, hence the enhanced OXPHOS. Therefore, we have revised the sentence in the manuscript since the lactate levels between wild-type PKM2 and PKM2-C326S are “similar”. We think that using the term "increase" may not be suitable for describing this minor distinction. We also

discussed this phenomenon in the Discussion (Line 526-548).

7) I have a question about the ectopic expression model. We agree that the MDA231 is a cancer cell line that already expresses high SSH-PKM2 due to the H2S synthesizing enzyme CBS and CTH. Why this cell model was used to overexpress PKM? Why not choose a cell line with low SSH PKM2? I suspect the original SSH-PKM in the cells will impact the function of the expressed form.

Response: We chose MDA-MB-231 cells as our major model primarily because breast cancer, especially TNBC, is highly dependent on glucose metabolism (PMID: 21498634; 26158266). Glucose metabolism is associated with tumor growth, metastasis, and drug resistance in breast cancer. We believe that blocking PKM2 sulfhydration can be considered as a metabolic therapy for breast cancer patients. Therefore, we used MDA-MB-231 cells as our model to proceed with this experiment.
During the revision process, we compared SSH-PKM2 levels across various breast cancer cell lines. Our findings revealed that the level of SSH-PKM2 in MDA-MB-231 is not higher compared to the other two breast cancer cell lines (MCF-7 and HCC-1395) we tested (Fig. 1H).

8) In Figure 6, the mouse number in this study was shown between 3-10, which means the mouse number is only 3 in some groups. I am questioning how the statistical analysis was conducted.

Response: For the mouse experiment, we conducted the experiment twice independently. In the initial trial, we analyzed data solely from the PKM2-wt and PKM2-C326S groups due to accidental deaths of mice in the PKM2-vector group at the animal facility during the experimental period. Subsequently, we performed a second experiment involving all three groups: PKM2-vector, PKM2-wt, and PKM2-C326S. Consequently, the total number of mice varied across groups: PKM2-vector (3 mice), PKM2-wt (9 mice), and PKM2-C326S (10 mice). We combined data from both experiments and conducted statistical analyses using two-way ANOVA followed by Tukey's multiple comparisons test for tumor growth curves and one-way ANOVA followed by post hoc test for all other analyses. The rate of tumor growth was significantly suppressed in the PKM2-C326S group in two independent experiments, in comparison to the PKM2-vector and PKM2-wt groups. Given the attained statistical power and in adherence to the Animal Protection Act mandating minimization of mice usage

in research, we decided not to proceed additional experiment.

9) It would be nice to show all the animal pictures instead of just 2 -3.

Response: All the animal pictures are shown in Figure S8 now.

10) The tumor growth experiment in Figure 6B showed that MDA231 cells expressing C326S mutation did not show any tumor growth, which contradicts the in vitro cell growth results in Figure 6A. This phenomenon or effect deserves further discussion.

Response: Thank you for your suggestions. We have added a paragraph to discuss the possible biological and experimental factors that could explain the differing response of the C326S mutant *in vitro* and *in vivo* (Line 466-484).

11) It was not discussed why the ectopic expression of wt-PKM2 did not affect metabolism, cell cycle, or cancer cell growth in vitro and in vivo in Figures 4, 5, and 6.

Response: We have discussed this aspect thoroughly in the Discussion (Line 485-500).

Reviewer #3 (Remarks to the Author):

Wang et al provide interesting evidence supporting the notion that sulfhydration of C326 in the M2 isoform of pyruvate kinase destabilizes the active tetrameric state of the enzyme and provides a mechanism of in vivo regulation of this key glycolytic enzyme. In this way, this PTM influences primary glucose metabolism and can promote tumorigenesis. The authors also present evidence that blocking the sulfhydration PTM at C326 through a C326S point mutation, leads to a more stable tetrameric enzyme assembly that in vivo inhibits tumour growth through alterations in glucose metabolism through stable PK activity that is insensitive to sulfhydration effects. The area of sulfhydration as a mechanism of regulatory post-translational modification (PTM) is an interesting and understudied area and the authors do a good job of presenting a comprehensive body of impactful work that suggests that modification of C326 may be an important in vivo regulatory mechanism of PK

that could impact cancer tumorogenesis.

While the general conclusions are largely supported by the data I do have some comments that I feel should be addressed by the authors as they relate to the interpretation and impact of specific results.

1) In general, I don't find that the evidence provided fully supports the notion that sulfhydration results in a shift in the dimer-tetramer equilibrium. In my view, the substitution leads to a more heterogeneous and dynamic population of states as the profiles are quite broad rather than a simple, stochastic shift in a population of states between the dimer and the tetramer. Further, the sizing data show that the WT in the absence of FBP populates the monomer/dimer state more than the treated enzyme in the presence of FBP begging the question of where is the data on the treatment of the enzyme in the absence of FBP? Perhaps I missed this data in my review but it would seem important to include. The authors state sulfhydration modulates the dissociation of PKM2 tetramers into dimers/monomers. Is it dimers or monomers? This distinction seems like an important one however as stated above, I don't feel the data supports the notion that a transition from one quaternary state to another is warranted as the data in my view suggest that the modification destabilizes the tetramer into a more complex/heterogeneous population of various oligomeric states and not a stochastic shift as the authors statements suggest. Further, the authors analyze the C326S mutant to state that it stabilizes the tetramer. Again, I don't think that this stochastic interpretation is supported by the data. Also, there appears to be an aggregate (large than tetramer) peak in the mutant that is ignored suggesting that a shift in the propensity to higher order states is also occurring. In general, I think a more thoughtful examination of the true effects of C326 modification and serine substitution and their influence on the nature of the enzymes quaternary state is warranted to be more consistent with the data that is provided.

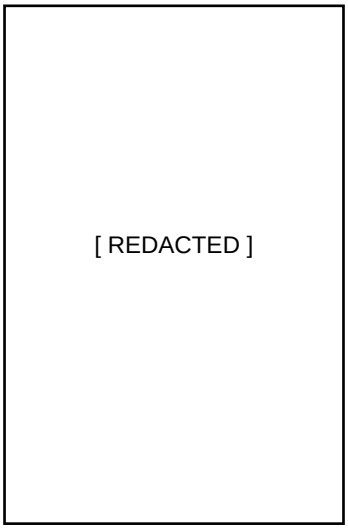
Response: We polished our purification of the recombinant proteins to eliminate the protein aggregation of PKM2 (large than tetramer) peak in the gel filtration analysis. The substitution of Cysteine 326 to Serine does lead to a higher ratio of the tetrameric form compared to wildtype PKM2 in vitro, as shown in our gel filtration assays with recombinant PKM2 (Fig. 2D). In cellular lysates, $PKM2^{C326S}$ exhibited a more heterogeneous and dynamic population (Fig. 2E). We attribute this observation to the complexity of PKM2 interacting with other

proteins inside the cell, which may obscure the evidence of stochastic shift from dimer to tetramer as we observed in the assay using recombinant PKM2 proteins. In sum, we believe that under simpler conditions as in the test tube, H_2S can convert PKM2 from tetramer into a monomer (Fig. S1D). However, in the much more complicated cellular environment, we are not certain if the sulfhydration of PKM2 interferes or facilitates its interaction with other proteins and how these interactions affect PKM2 oligomerization state. In the future, we will investigate the relationship between PKM2 sulfhydration and its interaction with other proteins through biochemical and structural biology approaches.

For Figure 1C, we now included the data of NaHS treatment alone.

2) The lack of recovery of full enzyme activity after DTT treatment (Fig 1B) needs to be explained by the authors. What is the general effect of NaSH treatment on the kinetic assay? The fact that WT activity is not fully recovered suggests that NaSH is causing other effects that reduce enzyme function or are irreversible or affecting other aspects/enzymes in the kinetic assay.

Response: Removal of the persulfide bond by DTT essentially rescued the activity of NaHS-treated PKM2 (Fig. 1B), aligning with the results that S-sulfhydration destabilize the tetramer (Fig. 1C). However, the recovery did not reach 100%. Similar observations of incomplete recovery after DTT treatment have been documented in studies of oxidized PKM2 (PMID: 22052977), suggesting that the reductive repair of oxidized PKM2 is not entirely effective. Considering that DTT is highly hydrophilic with 2-OH groups, it is possible that DTT cannot effectively penetrate the hydrophobic core of PKM2. Or the transition from the reduced dimer/monomer form to the tetrameric PKM2 may be slow. Longer incubation with DTT may resolve these issues for other enzymes but not for PKM2. This is because PKM2 activity decreases over time, limiting the effectiveness of extended DTT treatment in fully restoring activity.



3) While perhaps not physiologically relevant, from a mechanistic standpoint it

would be important to understand if the disruption in the quaternary state and the correlated activity changes by sulfhydration of C326 are specific to this modification or whether other chemical modifications of this residue result in similar effects. Related to this point, is modification to the sulfhydrated form necessary or can the general oxidation of this residue to sulfenic, sulfinic and sulfonic states accomplish the same functional effect? Lastly, I don't understand the statement that AOAA treatment supports the hypothesis that the effects at cysteine 326 are predominantly mediated through sulfhydration and not other modifications/general oxidation. Clarification of this point would be helpful.

Response: In the substitution experiment, it is indeed challenging to definitively ascertain whether the modification hindered by the mutation on C326 is solely sulfhydration or possibly other modifications. Nevertheless, our analysis detected sulfhydration specifically at C326 of endogenous PKM2 extracted from cell lysates (Table. S1), particularly in regard to irreversible modifications such as sulfinic acids (R−SOOH) and sulfonic acids (R−SOOOH). These modifications should be identifiable through mass spectrometry analysis if they occur but was not detected in our analysis. Regarding whether the sulfhydrated form is necessary or if the general oxidation of this residue to sulfenic, sulfinic, and sulfonic states can achieve the same functional effect, we cannot provide a definitive answer due to technical limitations in selectively modifying only one amino acid, like C326 in our case. Therefore, to strengthen our understanding of whether sulfhydration is crucial for PKM2-related functions, we addressed this question indirectly by inhibiting H_2S production using the H_2S inhibitor AOAA or by silencing H_2S-producing enzymes with siRNA. Our results demonstrated that treatment with AOAA or knockdown of CBS/CTH led to the inhibition of PKM2's nuclear localization (Fig. S1H, J), cytokinesis inhibition (Fig. S7C-G), as well as cell proliferation (Fig. 1F). Collectively, the outcomes from AOAA treatment and CBS/CTH knockdown further support the notion that the effects observed upon impeding modifications at cysteine 326 of PKM2 are predominantly mediated through sulfhydration. We have addressed the limitations of our experiment extensively in the Discussion section (Line 413-435).

4) It is unclear what the importance of understanding structural basis for the C326S mutation on tetramer stabilization is to the presented work. The mechanistic basis by which the serine substitution affects the oligomeric state

of the enzyme seems tangential to the rest of the work as it provides no insight into the real question that is not answered and that is how the modification of C326 via sulfhydration stimulates the opposite effect. It is also hard to rationalize how the single substitution is supporting an alternate tetrameric form.

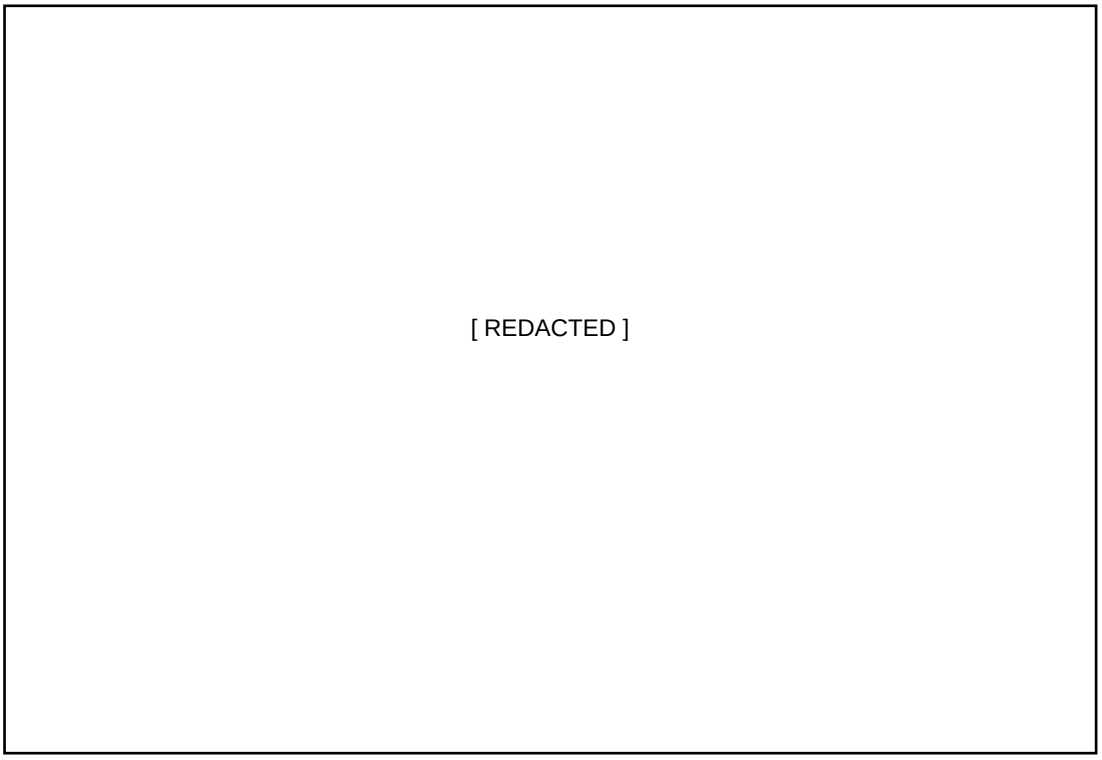
Response: We value the reviewer's concern regarding the direct relevance of the structural analysis of the C326S mutation in PKM2 to our study's broader focus on sulfhydration. While our primary aim was to elucidate the structural impact of sulfhydration on PKM2, we encountered challenges in obtaining the crystals of sulfhydrated PKM2. We thus explored the C326S mutation as an alternative approach to gain insight into how alterations at this residue affect PKM2's oligomeric state (Fig. S5). Through molecular simulations, we sought to understand the effects of sulfhydration at C326 on the tetrameric stability of PKM2. Our crystallography analysis combined with simulation helped us infer that while sulfhydration at C326 destabilizes the tetramer, replacing cysteine with serine at this position promotes a more stable tetrameric structure.

On a more basic level, I don’t understand the interpretation where the authors state that replacing the Cys with Ser at residue 326 of PKM2 remotely changes the dynamics of the C domain and promotes tetramerization (Fig. 3). If I am interpreting the structures correctly, the R and T structures compared to this new structure have ligands bound to the C domain where this structure is unligated. If this is the case, how do the authors distinguish between differences that reflect influences of the mutation versus the more likely conclusion that the differences in the disorder/order of this domain are the result of the impact of comparing ligated to unligated states of the enzyme and the order in the domain that is correlated with ligand/allosteric effector binding.

Response: We appreciate and understand the concern raised by the reviewer. To address this, we refer to two crystal structures of the apo PKM2 tetramer without bound activator or inhibitor: 1ZJH.pdb and 3SRH.pdb. These structures reveal highly similar or even identical conformations among the protomers. For instance, in 1ZJH.pdb, with a space group of I222 and one protomer in one asymmetry unit, the tetramer exhibits four identical protomer conformations. Similarly, in 3SRH.pdb, where one tetramer is present in one asymmetry unit, the structural overlay of the four protomers indicates very similar conformations. Furthermore, comparison of the protomers within 1ZJH and 3SRH also

highlights their significant similarity (see figure below).

These structural analyses suggest minimal dynamics in the unliganded state of PKM2. Therefore, the differences we observe can be attributed primarily to the introduced mutation rather than variations arising from comparisons between ligated and unligated enzyme states.



Lastly, meaningful assessment of the interpretation of the structural data is impossible without access to the model and map files. Further to this point, the PDB deposition report was not provided as a supplemental file.

Response: We appreciate the reviewer's suggestion. We included the model (8hgf.pdb and ADAD-tetramer.pdb) and map (8hgf-map.mtz) files, along with the PDB deposition report (8HGF_val-report-full.pdf) as supplemental files. For easy access, these files can also be downloaded directly from the Protein Data Bank (https://www.rcsb.org/structure/8hgf).

Minor Points

Several instances there is the statement the C326 can be a therapeutic target for anti-cancer drug development. I fail to see how a specific amino acid can

be targeted for therapeutic development beyond gene therapy. The authors need to provide more context to what approach they are referring to or remove these unsupported statements.

Response: In response to the reviewer's feedback, we have clarified the statement regarding C326 as a potential therapeutic target. Irreversible small molecular inhibitors targeting specific cysteine residues of proteins have been identified successfully in the past, such as those for the KRAS G12C mutant (Nature. 2013, 503, 548–551, PMID: 24256730) and PKM2 (J. Med. Chem. 2018, 61, 9, 4155–4164, PMID: 29641204). Notably, these inhibitors have shown promising therapeutic effects in clinical trials. To target the C326 of PKM2, one could explore nature-product-derived compounds capable of selectively influencing PKM2 through covalent binding at residue C326. We believe this clarification provides a more accurate representation of the potential therapeutic approach and appreciate the reviewer's valuable input.

The notion that pyruvate kinase (PK) regulates the final rate-limiting step of glycolysis by catalyzing the phosphoryl transfer from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to produce pyruvate and ATP is not universally supported. While this is a thermodynamically irreversible step in the process it is not necessarily the solely rate limiting process as reported most recently by Zuo J, Tang J, Lu M, Zhou Z, Li Y, Tian H, Liu E, Gao B, Liu T, Shao P. Glycolysis Rate-Limiting Enzymes: Novel Potential Regulators of Rheumatoid Arthritis Pathogenesis. Front Immunol. 2021

Response: We have revised our wording and cited the paper in the manuscript as suggested by the reviewer (Line 85-87).

On p.7 the authors state that FBP may dissociate into fructose by NaHS treatment. Are the authors intending to state that FBP is hydrolyzed into fructose and 2xPi? This statement should be clarified so it makes sense from a chemical standpoint.

Response: We agree that FBP will not hydrolyze into fructose and 2xPI when treated with NaHS. However, since this data is the groundbreaking results demonstrating that NaHS can destabilize PKM2 tetramerization, we believe it

is important to conduct a validation experiment to eliminate any potential factors that could influence the formation of the PKM2 tetramer by FBP. As a result, our validation data indicate that FBP remains unchanged in the presence of NaHS, supporting the idea that NaHS indeed destabilizes PKM2 tetramerization.

The authors state that the protein concentration was determined via absorbance at 280nm however they do not provide an extinction coefficient for that calculation.

Response: We thank the reviewer for pointing this out. The extinction coefficient of PKM2 at 280 nm, 29910 $M^{-1}cm^{-1}$, is now provided in the Materials and Methods.

The crystallographic data table is incomplete. For instance, it lacks statistics and validation parameters such as Molprobity and Ramachandran analysis. Further, some sections make no sense such as 14857 under the heading of macromolecules. I assume the authors mean there were 14857 atoms in the macromolecular portion of the structural model. Average B factors should be broken down for the different components of the model such as solvent, macromolecules, heteroatoms as is normal convention not just an overall average B factor reported.

Response: Thank the reviewer's suggestion to enhance the completeness of the crystallographic table. We have included the clash score, MolProbity score and Ramachandran analysis in the revised crystallographic data table (Table S2). We also have broken down the No. of Atoms and Average B factor. There is only the protein component in our PDB, so only values for protein are listed in this table.

What do S and X mean in Table S1? These terms should be described in the table footnote.

Response: We modified it by replacing the terms "V" and "X" with "Yes" and "No" in the "Detectable" column of Table S1.

There are some general English language/grammar issues throughout the manuscript in particular there is a notable lack of articles in many sentences. Careful editing of the work for these issues is recommended.

Response: We have meticulously revised the manuscript, and it has undergone review by an English language editing service. The editing certificate is provided below.

AJE

Editing Certificate

This document certifies that the manuscript

Hydrogen Sulfide Coordinates Glucose Metabolism Switch through Destabilizing Tetrameric Pyruvate Kinase M2

prepared by the authors

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List of Key Improvements from Revision:

Main Figures

1.	Added the data of NaHS treatment alone: **Fig. 1C**
2.	Cell proliferation rate in cells with CBS&CTH knockdown: **Fig. 1F**
3.	Two additional breast cancer cell lines, MCF-7 and HCC-1395, were included: **Figs. 1G, H**
4.	Refined gel filtration analysis of PKM2 wild-type and C326S: **Fig. 2D**
5.	Level of PKM2 and SSH-PKM2 in stable cell line: **Fig. 4A**

Supplementary Data

1.	Additional Experiments in cells with CBS&CTH knockdown: **Fig. S1F, G, J, K; Fig. S7G.**
2.	Additional Experiments in PC3 cancer cell line: **Fig. S1A, B; Fig. S2E, G, H; Fig. S7H**
3.	Additional Experiments in normal epithelial MCF-10A cell line: **Fig. S2D, F**
4.	Mass measurement of sulfhydrated PKM2 molecules by mass photometry: **Fig. S1D**
5.	H_2S detection in cells treated with AOAA: **Fig. S1E**
6.	Molecular dynamics simulation on sulfhydrated PKM2 tetramer: **Fig. S5; Table S3**
7.	Level of PKM2 in PC3 stable cell line: **Fig. S6A**
8.	All the tumor pictures are shown: **Fig. S8**
9.	Revise the text to make it easier to understand: **Table S1**
10.	The clash score, MolProbity score, and Ramachandran analysis are included: **Table S2**

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the majority of my concerns and have greatly improved the scientific quality and clarity of their manuscript. My only suggestion is to where they stated in their rebuttal that "The rescue experiment indicated that NaHS was only able to partially reverse the AOAA-induced reduction in cell proliferation (Data not shown)", that the authors do show this data (can be put in the supplemental data figure if needed). This is important as it helps the readers understand the broad impact of AOAA can be outside its inhibition of H2S production.

Reviewer #2 (Remarks to the Author):

The authors added some experiments to address most of my questions in this revised manuscript. For some of my concerns, the authors provided appropriate responses and explanations. I have only one suggestion here.

1) The author added PC-3, MCF7, and HCC1395 in this revision to generalize the findings identified in MDA 231 cells. Please provide some rationale for the selection of these cell lines. PC-3 is a prostate cancer cell. What are the typical characteristics this cell line shares with MDA231, a breast cancer cell line? In addition, the HCC1395 is a TNBC cell line similar to MDA231 cells. However, the MCF7 is a luminal breast cancer cell line that has a very different metabolic background. Are the results obtained in MCF7 consistent with those from TNBC cell lines? If yes, please explain.

Minor questions.
1) Please check the ENGLISH for the supplementary information, including the Figure legends. I found “Blockage” in multiple supplementary Figure legends. It seems “Blockade” is more accurate in the current scenario.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all of my prior concerns in their revised manuscript.

We thank the reviewers for dedicating their valuable time to review our work and providing insightful suggestions to enhance the manuscript's rigor and impact on cancer metabolism. In this final revised version, we have incorporated the data and words as suggested by the reviewers.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the majority of my concerns and have greatly improved the scientific quality and clarity of their manuscript. My only suggestion is to where they stated in their rebuttal that "The rescue experiment indicated that NaHS was only able to partially reverse the AOAA-induced reduction in cell proliferation (Data not shown)", that the authors do show this data (can be put in the supplemental data figure if needed). This is important as it helps the readers understand the broad impact of AOAA can be outside its inhibition of H2S production.

Response: Thank you for the suggestion. We have incorporated data showing that NaHS can only partially reverse the AOAA-induced reduction in cell proliferation (Fig. S1l) and discussed that AOAA not only inhibits H_2S-producing enzymes but also inhibits enzymes that require vitamin B6 as a coenzyme (Line 165-171).

Reviewer #2 (Remarks to the Author):

The authors added some experiments to address most of my questions in this revised manuscript. For some of my concerns, the authors provided appropriate responses and explanations. I have only one suggestion here.

1) The author added PC-3, MCF7, and HCC1395 in this revision to generalize the findings identified in MDA 231 cells. Please provide some rationale for the selection of these cell lines. PC-3 is a prostate cancer cell. What are the typical characteristics this cell line shares with MDA231, a breast cancer cell line? In addition, the HCC1395 is a TNBC cell line similar to MDA231 cells. However, the MCF7 is a luminal breast cancer cell line that has a very different metabolic background. Are the results obtained in MCF7 consistent with those from TNBC cell lines? If yes, please explain.

The reason we conducted experiments mostly on cell lines from two different cancer types, breast cancer and prostate cancer, is to demonstrate that the destabilization of PKM2 tetramers by H_2S is a general phenomenon occurring across various cell types in the body. We have added our rationale to the main text (Line 206-209).

Minor questions.

1) Please check the ENGLISH for the supplementary information, including the Figure legends. I found “Blockage” in multiple supplementary Figure legends. It seems “Blockade” is more accurate in the current scenario.

Response: Thank you for the suggestion. We have carefully reviewed the English and replaced “Blockage” with “Blockade” in the supplementary information.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all of my prior concerns in their revised manuscript.

Response: Thank you for your feedback and for reviewing our manuscript.