

Peer Review File

Hexosaminidase B-driven cancer cell-macrophage co-dependency promotes glycolysis addiction and tumorigenesis in glioblastoma



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript entitled “HEXB-driven cancer cell-macrophage co-dependency promotes glycolysis addiction and tumorigenesis in glioblastoma”, the authors investigated the metabolic enzyme hexosaminidase B (HEXB) in glioblastoma cells and macrophages during the development of glioblastoma (GBM). The authors showed that HEXB expression is associated with poor survival in GBM patients. They also showed HEXB promotes the proliferation and tumorigenicity in GBM. HEXB was found to be associated with integrin beta-1 (ITGB1) and integrin-linked kinase (ILK), leading to the activation of YAP1/HIF1a axis. HIF1a then upregulate HEXB expression, forming a positive feedback loop. HEXB also recruits macrophages to tumor tissues and promote the development of GBM. Further, HEXB expression is suppressed in IDH mutant cells via 2-HG dependent manner.

The roles of HEXB in the pathogenesis of GBM remain unclear. In this regard, the findings of the manuscript are potentially important. However, the reviewer has several concerns on this manuscript.

1. In this manuscript, the authors administered recombinant HEXB protein to in vitro cultured cells. Did the authors confirm that recombinant HEXB enter the cells? How does HEXB protein enter the cells? Intracellular or extracellular localization of HEXB can be examined in more detail.

2. As for the tumorigenic effects of HEXB, what are the roles of its enzymatic activity in the pathogenesis of GBM? Does gangliosides or other end-products of this enzyme contribute for the development of GBM?

3. HEXB is known to act as a homodimer or heterodimer complex with HEXA. It is unclear whether HEXB homodimer or heterodimer is acting to promote tumorigenicity in GBM.

HEXB has been reported to play a key role in the lysosomal function of macrophages. How is lysosomal function of macrophages affected in HEXB deficient cells? What are the roles of macrophage lysosomal function in the pathogenesis of GBM?

4. In figure 1E, colocalization rate of HEXB-CCR2 (M1 marker) is higher than that of HEXB-CX3CR1 (M2 marker). The results do not support the authors' conclusion that HEXB is predominantly expressed in M2 macrophages. How does HEXB recruit macrophages to the tumor tissues? The molecular processes remain unclear.

5. 2-HG is known to suppress HIF-prolyl hydroxylases, suppressor of HIF-1a signaling, leading to the accumulation of HIF-1a protein. Considering the finding that HEXB is induced by HIF1a (Figure 5I, J), HEXB expression is expectedly upregulated in IDH1 mutant cells.

6. In figure 1C, the authors performed transcriptome analysis and found that HEXB as one of the differentially expressed genes. The processes by which the authors analyzed the data must be explained in more detail. In addition, the number of genes in each step or column can be demonstrated.

Reviewer #2 (Remarks to the Author):

In this paper, the authors studied interplay between TAMs and GBM cell glycolysis. They nominated HEXB as a key molecule facilitating cross talk between TAMs and GBM cells through bulk and single cell RNA seq analysis. They showed that HEXB upregulates glycolysis in GBM cells. They also show that inhibition of HEXB leads to survival benefit in vitro and in mice. They propose that HEXB acts through ITGB1-ILK complex and promotes nuclear translocation of YAP-1. This, in turn, activates HIF1 α which further promotes transcription of HEXB and glycolytic enzymes. They propose that GBM derived HEXB induces chemotaxis of M2 TAMs which secrete more HEXB and this leads to enhanced glycolysis in GBM cells (TAMs-associated glycolysis-promoting network). This article has numerous strengths. The biology described in the paper is novel and of potential therapeutic importance. The authors' experimental design and narrative is very well thought out and logical. The models used are appropriate (both PDXs/GSCs and murine-derived GBMs). They perform rigorous testing of HEXB and its downstream biology in several ways: knockdown, pharmacological inhibition, addition of recombinant protein. All these modulations show consistent results and support their model. Further, they use excellent biochemical studies to identify the specific region and amino acid of HEXB3 that binds to ITGB1. They also intervene directly on the YAP1 with inhibitors and constitutively active models to show that it directly mediates the effects of HEXB. I anticipate this manuscript will have high impact on the field of cancer/immune-metabolism and neuro-oncology. I do have several comments that I hope will help clarify some of the results.

Major Comments:

1. Line 140- 144. "According to cross differential analysis of glioma cell intrinsic secretory protein encoding-genes associated with IDH1 status and TAMs polarization in bulk tumor and single cell RNA-sequencing cohorts (Fig.1C), HEXB was identified as a potential determinant for malignant cell glycolysis associated with TAMs (Supplementary Table S2)."

The details of nomination of HEXB are unclear. In fig 1C, the source of M1 vs M2 macrophages and monocyte vs macrophage circles in the venn diagram of cross differential analysis is not clear in the legend, text or the supplementary table. It would be helpful if the authors simplified this nomination or explained it in more detail.

2. Line 350-356: The co-injection model was not described in the methods, nor was any reference cited in the main text. Please provide a reference showing that the macrophages co-injected along with GBM cells survive and can therefore mediate the proposed effects on tumor growth. Alternatively, please show any IHC images that show higher abundance of M2 macrophages in the co-injected tumors when compared to GSC injection only tumors. Or if any labelling of macrophages was performed prior to injection, please show the labelled macrophages in the tumor.

Minor comments:

Line 279: Please provide reference for the statement “It was reported that YAP1 could bind with HIF1a to maintain its stability and further activate transcription of downstream oncogenic glycolytic genes (like PKM2) to enhance glycolytic activity.”

Line 330: Fig 6B shows positive correlation and co-localization of HEXB with M2 macrophage marker, CD163. Was CD80 or CD 86, which are markers for M1 macrophages, also tested for colocalization with HEXB to show that it does not show correlation?

The details of the Sleeping beauty- GSC model are not mentioned in the manuscript or supplementary material. There is also no reference to any previous publications by the authors using this model. Please provide a reference or the details of how the sleeping beauty model was developed.

Typographical errors:

There are a few typographical errors intermittently in the manuscript. Example:

Line 104: Sing-cell instead of single-cell

Line 191: Sleep beauty instead of sleeping beauty.

Reviewer #3 (Remarks to the Author):

Zhu et al compared gene expression in IDH-wildtype and mutant grade 4 gliomas using public RNA-seq data. They identified glycolysis as the main metabolic difference between the two indications, and a positive correlation with TAM content. They identify HEXB as correlating with this signature and note its expression in GBM cells and in macrophages. They found that perturbation of HEXB alters survival in vivo and that HEXB enhances glycolysis by activating YAP1 nuclear traslocation. The study is rigorously done and the conclusions are largely supported by the data. I would ask the authors to consider the following points.

The authors cite prior reports of TAMs constituting 30%-50% of the cellular tumor. However, more recent assessments would place this figure closer to 10%-15% in human GBMs

The authors mention they used “well-recognized” macrophage and glycolysis scores as input to theTimer method. Please specify the source and justification for these scores in the supplement.

The survival analysis of supplementary fig 1k-l or figure 7E-F do not appear to be controlled for IDH mutation status, age or sex.

Many of the results are obtained from the use of GAL-PUGNAC which is an OGA inhibitor. Can the authors provide evidence that this compound directly targets HEXB?

The authors show that genetic perturbation of HEXB modulates survival in xenografts and that GAL-PUGNAC treatments prolongs survival in xenografts and in syngeneic SB-based models. Does genetic inhibition of HEXB in the syngeneic SB model enhance survival? Over-expression? Does this depend on adaptive and/or innate immunity? The studies in immunocompetent mice are all done with Gal-P but not with genetic manipulation and as such it is not clear if modulation of HEXB is

causing the differences in and to what extent this depends on immune infiltrates. In figure 2A-B the authors note statistical significance, but there are no replicates of the non-targeting control, similarly for figure 5A-D.

Reviewer #4 (Remarks to the Author):

The manuscript “HEXB-driven cancer cell-macrophage co-dependency promotes glycolysis addiction and tumorigenesis in glioblastoma” identifies HEXB as a regulator for glycolysis in IDH1 wildtype glioblastoma, and reports HEXB impacts TAMs recruitment and polarization. A strength of this manuscript is that it investigates both the molecular mechanism of HEXB’s action and the therapeutic potential of targeting HEXB, providing a large amount of data. However, a general issue is that specific methods and exact controls for many experiments are not clearly described, making it difficult to review the scientific merits of these experiments. The writing of the manuscript needs to be improved as well. Some specific comments are below.

1. It is unclear what exact result is being analyzed in Figure 1A-B and how the genesets are defined. It appears to be RNAseq of tumor samples, but unclear whether it is bulk RNAseq or single cell RNAseq, and how is the data normalized. If it is bulk RNAseq, the correlation between “glycolysis score” and “macrophage score” (again, the definition of these are not clear) can be explained by that in some samples, macrophage percentage is higher, and macrophage tends to be more glycolytic than other cell types, such as GBM, rather than glycolysis in IDH1 WT GBM is higher, *and* it is associated with more TAMs. This is important because there is some evidence in the literature that high glycolysis, or Warburg effect, observed in solid tumor is largely driven by the immune cells in the tumor rather than cancer cells. (e.g. Reinfeld et al, Nature 2021)
2. It is unclear how is “glioma intrinsic gene” defined. If it is from ssRNA seq, the exact method needs to be better described. From the results later, it seems that not only glioma cells express HEXB. It would be helpful to assess what percentage of HEXB in tumor microenvironment is GBM cells -originated. To answer this question, it would also be helpful to understand what portion of the tumor is GBM cells, and TAMs. Flow or ssRNA-seq can give this result.
3. Detail is also needed for seahorse experiments. How long was the cells in seahorse plate? Given that HEXB is a secretory protein, if the cells are just seeded with fresh media, HEXB is unlikely to accumulate to meaningful amount in the extracellular space. So what is the mechanism? If the cells are seeded for some time to allow HEXB accumulation (a measurement of HEXB level in media would be helpful), then how is the impact of different proliferation rates adjusted? Are the seahorse results normalized to live cell number in each condition?
4. The claim that “TAMs recruited by cancer-HEXB feedback enhances GBM cells glycolysis” is not well supported. Specific evidence is needed to show that recruited TAMs directly promote glycolysis in GBM cells. The mechanism by which TAMs could enhance GBM cells glycolysis is also unclear.
5. In the HIF section, the authors claimed that “HIF stability is reduced with YAP1 inhibition”.

However, it is unclear how Figure 5 measures HIF “stability” rather than level.

6. To validate the pro-glycolytic effect of HEXB is through HIF, it would be clearer to directly report the transcript levels of specific HIF target genes in glycolysis, and how they are affected when HIF is silenced, and whether their expression in shHEXB can be rescued when cells are treated with HIF stabilizer. It is not very clear how “index of HIF transcriptional activity” was defined or measured.

7. In macrophage migration assay, the potential difference in macrophage survival and proliferation during the 22h period needs to be controlled for.

Minor points

1. Figure 1D, the label is unclear what is being probed. For “IDH WT”, the representative image is labeled “IDH1 R132H”, is that a mislabel?

2. There are some grammatical errors and typos throughout. For instance, in the very first sentence of abstract, the word “of” before “cancer” seems to be an error.

3. It is unclear what the authors mean by “TAMs-associated glioma cell glycolysis”. Some phrasing in this manuscript is confusing.

Point by point response to the reviewers' comments:

Response: We really appreciated the reviewers for their constructive and insightful comments on our work. We hope that the point-by-point responses listed below and the revised manuscript could address the comments and questions of each reviewer (Reviewers' comments are in *italic*). All changes to the initial submission are reported here and highlighted in the accompanying revised manuscript in yellow color.

Reviewer #1 (Remarks to the Author):

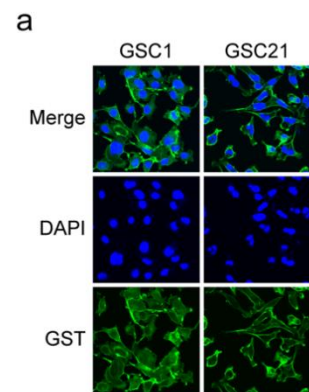
In this manuscript entitled "HEXB-driven cancer cell-macrophage co-dependency promotes glycolysis addiction and tumorigenesis in glioblastoma", the authors investigated the metabolic enzyme hexosaminidase B (HEXB) in glioblastoma cells and macrophages during the development of glioblastoma (GBM). The authors showed that HEXB expression is associated with poor survival in GBM patients. They also showed HEXB promotes the proliferation and tumorigenicity in GBM. HEXB was found to be associated with integrin beta-1 (ITGB1) and integrin-linked kinase (ILK), leading to the activation of YAP1/HIF1 α axis. HIF1 α then upregulate HEXB expression, forming a positive feedback loop. HEXB also recruits macrophages to tumor tissues and promote the development of GBM. Further, HEXB expression is suppressed in IDH mutant cells via 2-HG dependent manner. The roles of HEXB in the pathogenesis of GBM remain unclear. In this regard, the findings of the manuscript are potentially important. However, the reviewer has several concerns on this manuscript.

Response: We deeply appreciate your constructive comments, which substantially improve the quality of current work.

1. In this manuscript, the authors administered recombinant HEXB protein to in vitro cultured cells. Did the authors confirm that recombinant HEXB enter the cells? How does HEXB protein enter the cells? Intracellular or extracellular localization of HEXB can be examined in more detail.

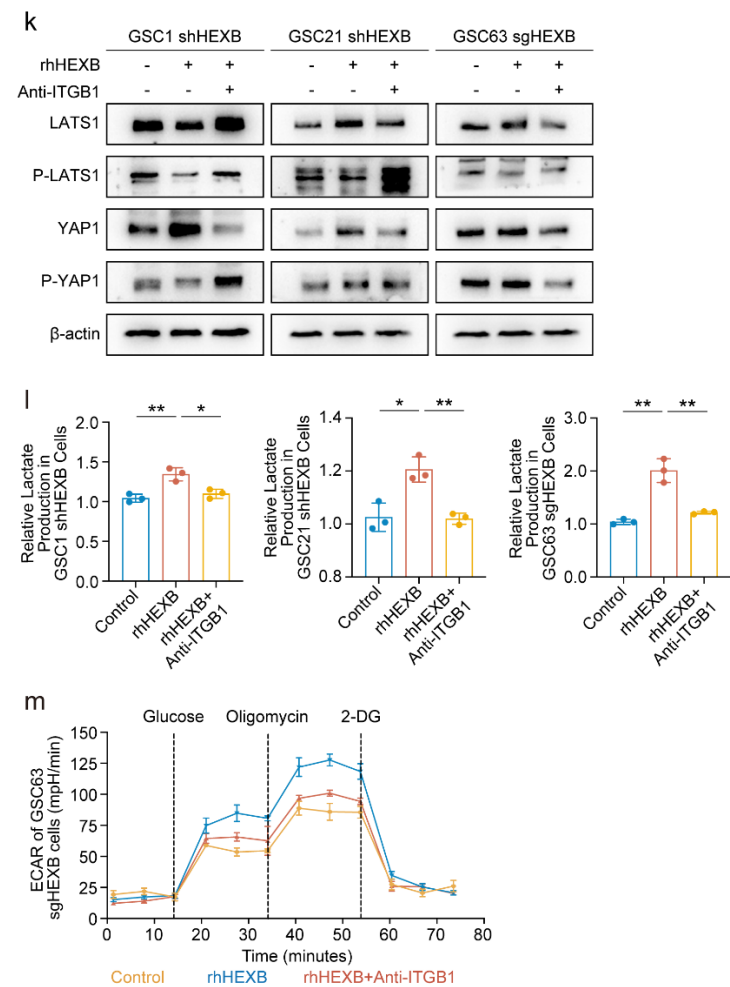
Response: We greatly appreciate your particularly professional feedback. Actually, HEXB exerts a series of functions on the surface of cell membrane in an exocrine form, which was validated by the following assays during the revision. Firstly, after treating GBM cells (GSC1 and GSC21) with GST-tagged HEXB recombinant protein for 48 hours, we fixed these cells and performed immunofluorescence detection of GST, presenting that GST-HEXB was mainly localized on the cell membrane (New Supplementary Fig. 13a). Secondly, since HEXB interacted with ITGB1 to perform its activities, we applied a human ITGB1 neutralizing antibody (R&D MAB17781) on the basis of administering human recombinant HEXB protein (rhHEXB). The result demonstrated that after ITGB1 blocking, the effect of rhHEXB on the downstream YAP1-HIF1 α signaling axis activation also significantly disrupted (New Fig. 4k). Meanwhile, the enhanced lactate production and glycolytic phenotype of GBM cells induced by rhHEXB was significantly restrained by the supplement of ITGB1 neutralizing antibody (New Fig. 4l and m). These results indicated that exogenous recombinant HEXB protein bound with ITGB1 on the cell membrane of GBM cells to activated its downstream signaling, rather than entering the cytoplasm. We have supplemented these data to the revised manuscript (New Supplementary Fig. 13a and New Fig. 4k-m) and edited the text accordingly (Page 11-12, line 312-315 and Page 12-13, line 343-348).

New Supplementary Fig. 13a, related to Fig. 4



New Supplementary Fig. 13a: The immunofluorescence staining of indicated GBM cells treated with GST-recombinant human HEXB. (Green: GST; Blue: DAPI).

New Fig. 4k-m



New Fig. 4

k Western blotting analysis of indicated proteins related to Hippo signaling pathway in indicated

GBM cell samples transduced with shHEXB or sgHEXB vector, and then treated with HEXB recombinant protein or ITGB1 neutralizing antibody.

l The analysis of lactate production in indicated GBM cell samples transduced with shHEXB or sgHEXB vector, and then treated with HEXB recombinant protein or ITGB1 neutralizing antibody (n = 3, t-test).

m The analysis of glycolytic rate (ECAR) of indicated GBM cell samples (GSC63) transduced with sgHEXB vector and then treated with HEXB recombinant protein or ITGB1 neutralizing antibody (n = 3, t-test).

*2. As for the tumorigenic effects of HEXB, what are the roles of its enzymatic activity in the pathogenesis of GBM? Does **gangliosides or other end-products of this enzyme** contribute for the development of GBM?*

Response: We truly appreciate this comment to help us further clarify the tumorigenic effect of HEXB. HEXB could promote the transformation of GM2 ganglioside to GM3 ganglioside. Previously, it has been reported that GM3 ganglioside serves as a protective factor for glioma and inhibits proliferation and invasion of glioma cells²³. Similarly, we observed that the treatment of sodium ganglioside GM3 (50 µg/ml and 100 µg/ml) on GSCs didn't significantly promote the proliferation of GSCs (New Supplementary Fig. 12a). Meanwhile, according to a previous report²⁴, GM2 activator (GM2A) was the cofactor of HEXB and directly involved in the transformation and metabolism of ganglioside GM2 to GM3. Thus, we knocked down GM2A with siRNA in GSCs and observed that the proliferation and lactate production of GSCs were not significantly affected by the knockdown of GM2A (New Supplementary Fig. 12b-d). These observations support that HEXB facilitate the development of GBM independent of its end-products, GM3. In addition, the specific performance was that the tumor promoting effect of HEXB was significantly restrained by the application of HEXB inhibitor Gal-PUGNAc (Gal-P), accompanied with significantly decreased HEXB protein expression (Previous Supplementary Fig. 6A upper panel). The activation effect of HEXB on glycolysis process and downstream signal axis was significantly weakened after the inhibition of its enzymatic activity by Gal-P (Previous Fig. 2c-e and 3f). Collectively, the above results support that the tumor promoting effect and downstream pathway activation of HEXB depends on its enzyme activity, but is independent of its end-product, GM3. We have added these new data to the revised manuscript (New Supplementary Fig. 12a-d) and edited Results section as follows:

Results (Page 11, line 291-307)

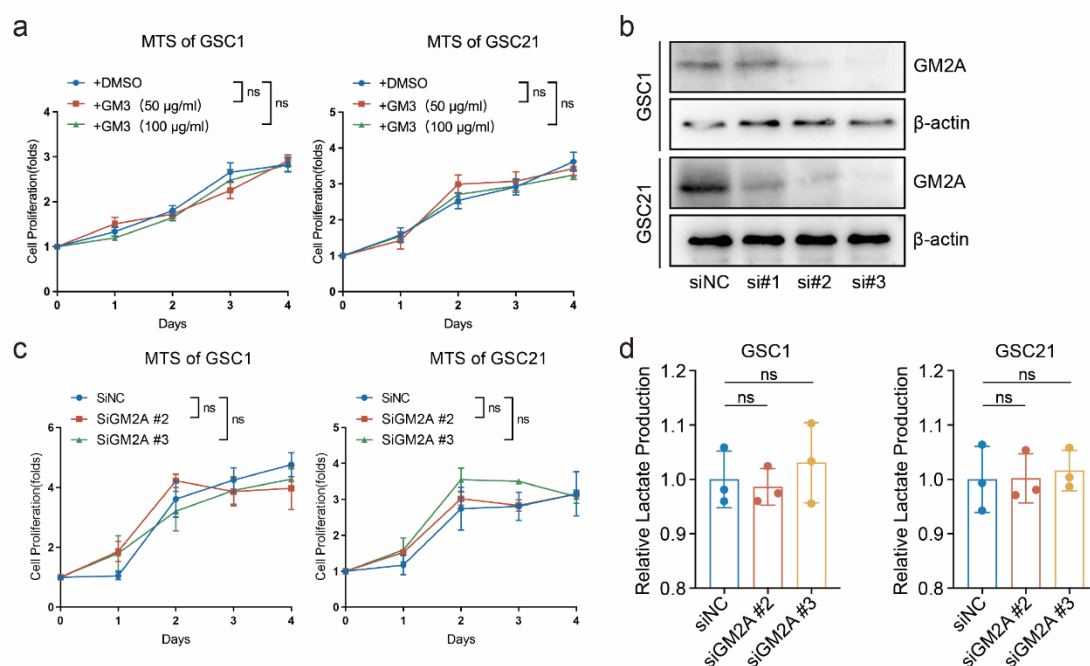
HEXB could promote the transformation of GM2 ganglioside to GM3 ganglioside. Previously, it has been reported that GM3 ganglioside serves as a protective factor for glioma and inhibits proliferation and invasion of glioma cells²³. Similarly, we observed that the treatment of sodium ganglioside GM3 (50 µg/ml and 100 µg/ml) on GSCs didn't significantly promote the proliferation of GBM cells (Supplementary Fig. 12a). Meanwhile, a previous report²⁴ demonstrated that GM2 activator (GM2A) was the cofactor of HEXB and directly involved in the transformation and metabolism of ganglioside GM2 to GM3. Thus, we knocked down GM2A with siRNA in GBM cells and observed that the proliferation and lactate production of GBM cells were not significantly affected by siGM2A (Supplementary Fig. 12b-d). These observations support that HEXB facilitates the development of GBM independent of its end-products, GM3. Moreover, the tumor promoting

effect of HEXB was significantly restrained by the application of HEXB inhibitor Gal-P, and the activation effect of HEXB on glycolysis process and downstream signal axis was significantly weakened after inhibiting its enzymatic activity by Gal-P (Fig. 2c-e and 3f). Collectively, the above results support that the tumor promoting effect and downstream pathway activation of HEXB in GBM cells depends on its enzyme activity, but is independent of its end-products GM3.

References

23. Fujimoto Y, et al. Ganglioside GM3 inhibits proliferation and invasion of glioma. Journal of neuro-oncology 71, 99-106 (2005).
24. Deschenes NM, et al. Characterization of a phenotypically severe animal model for human AB-Variant GM2 gangliosidosis. Frontiers in molecular neuroscience 16, 1242814 (2023).

New Supplementary Fig. 12a-d, related to Fig. 3



New Supplementary Fig. 12. GM3 and GM2A are not essential for promoting the proliferation and lactate production of GBM cells.

a MTS assays indicating that the proliferation ability of indicated GBM cells (Left: GSC1, and right: GSC21) was not significantly restrained by indicated concentrations of GM3 treatment (50 ng/ml and 100 ng/ml; n = 3, t-test).

b Western blotting analysis of indicated GBM cells (GSC1 and GSC21) transduced with indicated siGM2A or non-targeted vectors.

c, d MTS (c) and lactate production analysis (d) of indicated GBM cells (Left: GSC1, and right: GSC21) transduced with indicated siGM2A or non-targeted vectors (n = 3, t-test).

3. *HEXB* is known to act as a homodimer or heterodimer complex with *HEXA*. It is unclear whether *HEXB* homodimer or heterodimer is acting to promote tumorigenicity in GBM. *HEXB* has been reported to play a key role in the lysosomal function of macrophages. How is lysosomal function of

macrophages affected in HEXB deficient cells? What are the roles of macrophage lysosomal function in the pathogenesis of GBM?

Response: We thank for this thoughtful comment, which help us improve the preciseness of the logic of our work. Previous reports disclosed that the *HEXA* and *HEXB* gene separately encodes the α and β subunit of hexosaminidase and HEXA protease is heterodimer complexes composed of α - β subunits, in contrast to HEXB protease, which is homodimers composed of β - β subunits^{18, 19}. In current study, the immunohistochemical detection of HEXA and HEXB protein in glioma tissue samples disclosed that the expression of HEXB protein in GBM was significantly higher than IDH1 mutant glioma (Revised Fig. 1d), while the expression of HEXA protein had no significant difference between GBM and grade IV IDH1 mutant glioma (New Supplementary Fig. 4a). This supported HEXB homodimer as the main form of hexosaminidases in GBM. Simultaneously, we conducted corresponding intervention and phenotypic detection of HEXA with the application of recombinant HEXA protein and HEXA knockdown. We observed that HEXA knockdown and the administration of recombinant HEXA protein has no significant regulatory effects on the lactate production and proliferation of GSCs (New Supplementary Fig. 7a-d). Combined with the observations on strong promoting role of HEXB on GBM glycolysis (Previous Fig. 2c-e), it is reasonable to infer that hexosaminidase mainly exerts its tumor promoting effect in GBM through its homodimer form, HEXB.

Our study mainly focused on the role of HEXB in glycolysis and malignancy of GBM cells, as well as the effect of tumor-secreted HEXB on TAMs. In previous manuscript, we did not address the role of HEXB in TAMs and its effect on the lysosomal function in TAMs. Therefore, according to your comment to extend the observation on the role of HEXB in promoting GBM progression, we employed THP-1 derived macrophage model to examine the lysosome function of macrophages with disrupted HEXB expression. We detected Cathepsin L Activity in THP1-derived macrophages transduced with HEXB knockdown or control vectors and performed western blotting analysis of HEXB and lysosome-functional markers including Lamp1, Lamp2 and ATP6V1H in these cells. The result demonstrated that disrupting HEXB expression in THP1-derived macrophages didn't significantly disturb Cathepsin L Activity and the above lysosome marker expression in these cells, which didn't support HEXB deficiency impacting the lysosome functions in macrophages (Reviewer only Fig. 1). Based on these observations, we have revised the Result section as follows:

Result (Page 8-9, line 208-223)

Besides, according to previous reports^{18, 19}, the *HEXA* and *HEXB* gene separately encoded the α and β subunit of hexosaminidase and HEXA protease was heterodimer complex composed of α - β subunits, in contrast to HEXB protease, which was homodimer composed of β - β subunits. The immunohistochemical detection of HEXA and HEXB protein in glioma tissue samples disclosed that the expression of HEXB protein in GBM was significantly higher than grade IV IDH1 mutant glioma (Fig. 1d and Supplementary Table 5), while the expression of HEXA protein had no significant difference between GBM and grade IV IDH1 mutant glioma (Supplementary Fig. 6). This supported HEXB homodimer as the main form of hexosaminidases in GBM. In addition, we conducted the intervention and phenotypic detection of HEXA with the application of recombinant HEXA protein and HEXA knockdown. We observed that HEXA knockdown and the administration of recombinant HEXA protein had no significant regulatory effect on the lactate production and proliferation of GBM cells (Supplementary Fig. 7). Therefore, it is reasonable to infer that

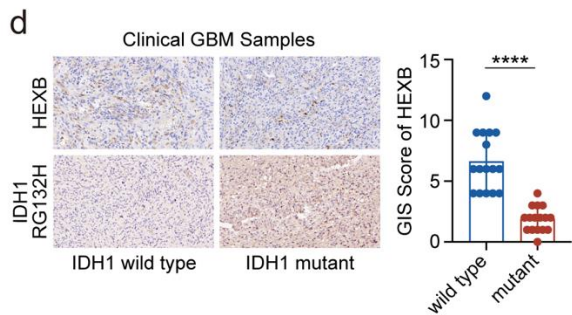
hexosaminidase mainly exerts its tumor promoting effect in GBM through its homodimer form, HEXB.

References

18. Montgomery MK, Taddese AZ, Bayliss J, Nie S, Williamson NA, Watt MJ. Hexosaminidase A (HEXA) regulates hepatic sphingolipid and lipoprotein metabolism in mice. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 35, e22046 (2021).

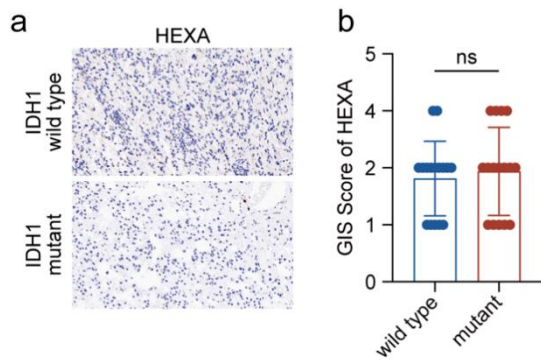
19. Shaimardanova AA, et al. Increasing β -hexosaminidase A activity using genetically modified mesenchymal stem cells. Neural regeneration research 19, 212-219 (2024).

Revised Fig. 1d



Revised Fig. 1d: Representative IHC images (Left) and analysis (Right) of HEXB staining in clinical grade IV IDH1 wild type (GBM) and mutant glioma tissue samples (n = 32, including IDH wild type n = 16 and IDH mutant n = 16; t-test).

New Supplementary Fig. 6, related to Fig. 1



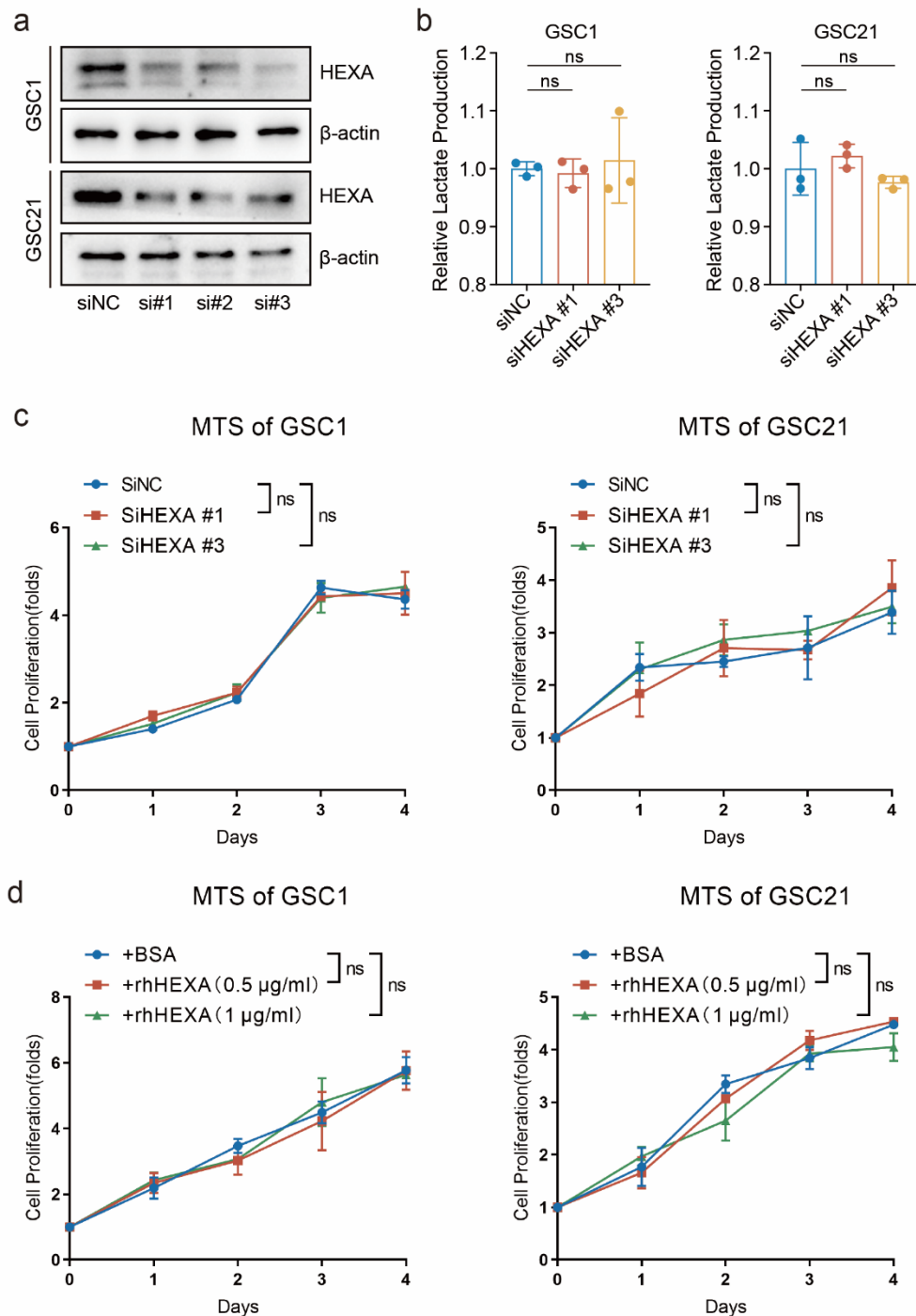
New Supplementary Fig. 6. The IHC staining analysis of HEXA in clinical IDH1 wild type (GBM) and grade IV IDH1 mutant glioma tissue samples.

a Representative IHC images of HEXA staining in clinical grade IV IDH1 wild type (GBM) and IDH1 mutant glioma tissue samples.

b The analysis of HEXA in grade IV IDH1 wild type (GBM) and IDH1 mutant glioma tissue samples (n = 32, including IDH wild type n = 16, IDH mutant n = 16; t-test).

(ns $P \geq 0.05$)

New Supplementary Fig. 7, related to Fig. 2



New Supplementary Fig. 7. HEXA is not essential for the lactate and proliferation of GBM cells.

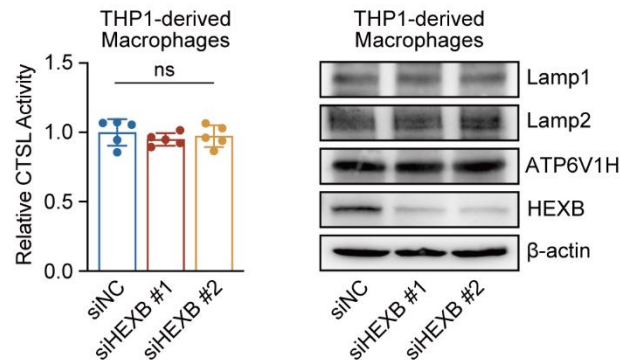
a Western blotting analysis of indicated GBM cell samples transduced with siHEXA or control vector, respectively.

b, c The lactate production analysis (b) and MTS assays (c) of GSC1 and GSC21 samples after transduction of siHEXA or control vector, respectively (n = 3, t-test).

d MTS assays of GSC1 and GSC21 samples with indicated BSA or recombinant human HEXA (rhHEXA) treatment (24h, 0.5 and 1 μ g/ml; n = 3, t-test).

(ns $P \geq 0.05$).

Reviewer only Fig. 1



Reviewer only Fig. 1

Left The detection of cathepsin L activity in THP1-derived macrophages transduced with HEXB knockdown or negative control vector (n = 5, t-test).

Right Representative western blotting images of HEXB and indicated lysosome-functional markers (Lamp1, Lamp2 and ATP6V1H) in THP1-derived macrophages transduced with HEXB knockdown or negative control vector.

4. In figure 1E, colocalization rate of HEXB-CCR2 (M1 marker) is higher than that of HEXB-CX3CR1 (M2 marker). The results do not support the authors' conclusion that HEXB is predominantly expressed in M2 macrophages. How does HEXB recruit macrophages to the tumor tissues? The molecular processes remain unclear.

Response: We appreciate this thoughtful comment. We used CCR2 as a marker of macrophages from peripheral blood. There are previous literatures supporting that CCR2 serves as a marker of macrophages from peripheral blood and its expression is significantly up-regulated in M2 macrophages, while CX3CR1 could be applied as a marker of inherent microglia in glioma (*Nat Neurosci.* 2019. **PMID: 30962630**; *Cancer Res.* 2017. **PMID: 28235764**). As shown in revised Fig. 1f, tissue polychromatic fluorescence confirmed that HEXB was more frequently localized in glioma cells (GFAP⁺ cells) and macrophages from peripheral blood (CCR2⁺ cells). To address this concern, we further improved the polychromatic immunofluorescence staining of glioma specimens by supplementing the detection of M1/M2 macrophage markers (M1: CD86⁺ cells; M2: CD163⁺ cells). As shown in Revised Fig. 1f, HEXB was more frequently expressed in M2 TAMs (CD163⁺ cells), instead of M1 TAMs (CD86⁺ cells). This data supports that the expression of HEXB in non-malignant cells predominantly located in M2 TAMs. We have revised Fig.1 and edited the corresponding text in Results section (Page 8, line 200-205).

Next, according to this thoughtful comment, we explored the potential molecular mechanism by which HEXB recruit macrophages to tumor tissues. Since HEXB is a molecule with function serving as a secretory protein, we first hypothesize that GBM cells secreted HEXB was involved in the recruitment of TAMs. Indeed, the migration ability of THP-1 derived macrophages was significantly enhanced by the addition of HEXB recombinant protein, which could be efficiently disrupted by the supplement of ITGB1 neutralizing antibody (New Supplementary Fig. 15j; n = 3, t-test). Secondly, Co-IP experiment disclosed that ITGB1 could also bind with HEXB in

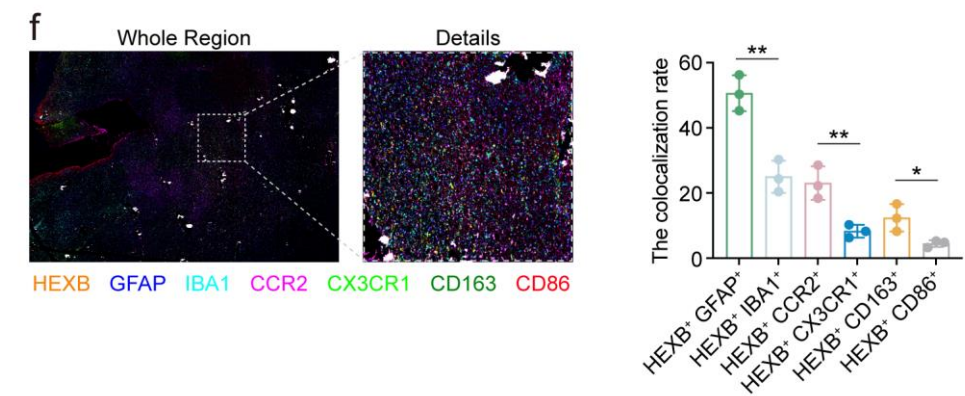
macrophages (New Supplementary Fig. 15i). The above results support that HEXB recruit macrophages through ITGB1 receptor on macrophages. We have revised Results section accordingly (Page 16, line 469-475).

References

PMID: 30962630. Takenaka MC, et al. Control of tumor-associated macrophages and T cells in glioblastoma via AHR and CD39. *Nature neuroscience* 22, 729-740 (2019).

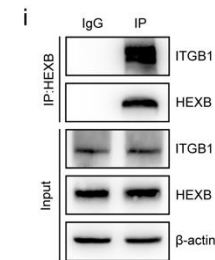
PMID: 28235764. Chen Z, et al. Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma. *Cancer research* 77, 2266-2278 (2017).

Revised Fig. 1f



Revised Fig. 1f: Representative multi-color immunofluorescence co-staining images (Left and middle) and analysis (Right) of HEXB, IBA1, CCR2, CD86, CD163, and GFAP in clinical GBM tissue samples.

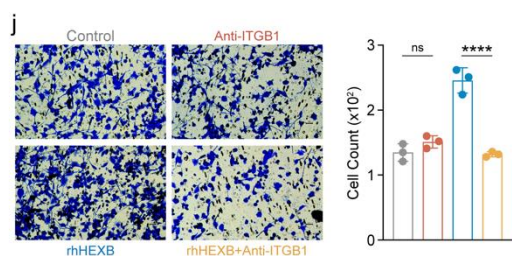
New Supplementary Fig. 15i, related to Fig. 6



New Supplementary Fig. 15i

i Co-IP analysis of HEXB and ITGB1 interaction in THP1-derived macrophages revealed that HEXB could interact with ITGB1.

New Supplementary Fig. 15j, related to Fig. 6



New Supplementary Fig. 15j

j The migration ability analysis of THP-1 derived macrophages with indicated treatment (Transwell assays; n = 3, t-test).

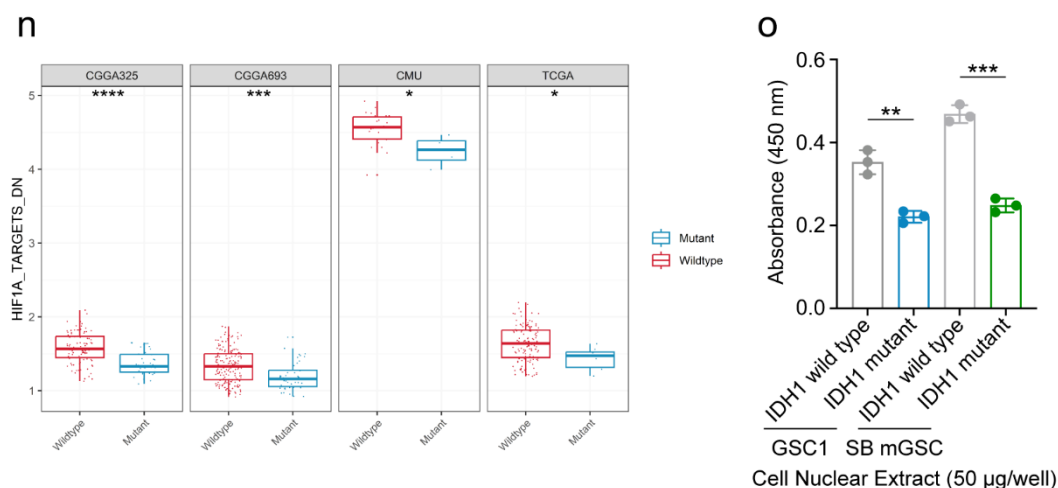
5. 2-HG is known to suppress HIF-prolyl hydroxylases, suppressor of HIF-1 α signaling, leading to the accumulation of HIF-1 α protein. Considering the finding that HEXB is induced by HIF1 α (Figure 5I, J), HEXB expression is expectedly upregulated in IDH1 mutant cells.

Response: We appreciated this thoughtful comment. According to early published report (*Science*. 2009. **PMID: 19359588**), the accumulation of 2-HG caused by IDH1 mutation could weaken the activity of HIF prolyl hydroxylases and then cause the accumulation of HIF1 α protein. However, there are studies revealing that activation of the HIF1 α pathway is not primarily regulated by R132H-mutated IDH1, but more likely via other established mechanisms such as hypoxia, transcriptional regulation and major genetic and epigenetic alterations resulting in oncogene gain of function or tumor suppressor gene loss of function (*Acta Neuropathol*. 2011. **PMID: 21181477**) and 2-HG may lead to the degradation of HIF1 α with stimulating PHD activity (*Nature*. 2012. **PMID: 22343896**). Furthermore, compared to IDH1 wild type tumors, IDH1 mutant glioma cells exhibits under-expressed LDHA, the key glycolytic enzyme and several other glycolysis essential genes, supporting that IDH1 mutant gliomas have limited glycolytic capacity (*Neuro Oncol*. 2014. **PMID: 24366912**) and IDH1 wild type glioma exhibited an elevated HIF1 α activity and expression than IDH1 mutant tumors (*Neuro Oncol*. 2019. **PMID: 31066901**). To further validate our conclusion, we performed the following analysis and experiments. Firstly, a HIF1 α score was established with the downstream target genes of HIF1 α (ELVIDGE_HIF1A_TARGETS_DN from GSEA; https://www.gsea-msigdb.org/gsea/msigdb/cards/ELVIDGE_HIF1A_TARGETS_DN.html) and ssGSEA method to evaluate the activation status of HIF1 α signaling pathway in glioma, according to CMU, CGGA and TCGA glioma datasets (New Supplementary Fig. 14n). The results demonstrate a consistent significant elevation of HIF1 α score in IDH1 wild type glioma than IDH1 mutant tumor, which implicates a higher activation of HIF1 α signaling pathway in IDH1 wild type glioma. Secondly, we acquired IDH1 wild type and mutant mGSCs from mouse spontaneous glioma samples derived from IDH1 wild type and mutant Sleeping Beauty (SB) transposon vectors, respectively. As shown below, western blotting analysis demonstrated that the expression level of HIF1 α in IDH1 wild type mGSCs were significantly higher than that in IDH1 mutant mGSCs (New Supplementary Fig. 17b). Thirdly, the transcriptional activity of HIF1 α in IDH1 wild type glioma cells were significantly higher than that in IDH1 mutant glioma cells (New Supplementary Fig. 14o). Together, these data support higher activation of HIF1 α in IDH1 wild type than IDH1 mutant glioma cells. We have supplemented these data to the revised manuscript and edited the text in the Results section accordingly (Page 14-15, line 401-426).

References

- PMID: 19359588.** Zhao S, et al. Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1 α . *Science* (New York, NY) 324, 261-265 (2009).
- PMID: 21181477.** Williams SC, Karajannis MA, Chiriboga L, Golfinos JG, von Deimling A, Zagzag D. R132H-mutation of isocitrate dehydrogenase-1 is not sufficient for HIF-1 α upregulation in adult glioma. *Acta neuropathologica* 121, 279-281 (2011).
- PMID: 22343896.** Koivunen P, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. *Nature* 483, 484-488 (2012).
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- PMID: 31066901.** Yao J, et al. Metabolic characterization of human IDH mutant and wild type gliomas using simultaneous pH- and oxygen-sensitive molecular MRI. *Neuro-oncology* 21, 1184-1196 (2019).

New Supplementary Fig. 14 n and o, related to Fig. 5

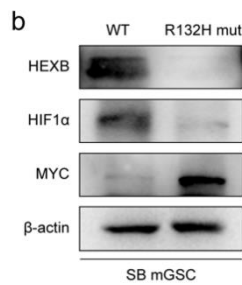


New Supplementary Fig. 14

n The analysis of HIF1 α downstream signaling pathway activation in IDH1 wild type and IDH1 mutant grade IV glioma based on a HIF1A score established by ssGSEA method according to a HIF1 α downstream target gene set (ELVIDGE_HIF1A_TARGETS_DN, https://www.gsea-msigdb.org/gsea/msigdb/cards/ELVIDGE_HIF1A_TARGETS_DN.html) in indicated glioma cohorts (CMU cohorts n = 30 (IDH1 wild type, n = 26; IDH1 mutant, n = 4), CGGA325 n = 139 (IDH1 wild type, n = 98; IDH1 mutant, n = 41), CGGA693 n = 239 (IDH1 wild type, n = 190; IDH1 mutant, n = 49), TCGA n = 148 (IDH1 wild type, n = 139; IDH1 mutant, n = 9), t-test).

o The analysis of HIF1 α transcriptional activities in indicated IDH1 wild type and mutant glioma cell samples (n = 3, t-test).

New Supplementary Fig. 17b, related to Fig. 5



Revised Supplementary Fig. 17b: Representative western blotting images of HEXB and HIF1 α in IDH1 wild type and IDH1 mutant mGSCs derived from mouse GBM tumors established by IDH1 wild type and mutant Sleeping Beauty (SB) transposon vectors, respectively.

6. In figure 1C, the authors performed transcriptome analysis and found that HEXB as one of the differentially expressed genes. The processes by which the authors analyzed the data must be explained in more detail. In addition, the number of genes in each step or column can be demonstrated.

Response: We appreciated this thoughtful suggestion and apologized for the inadequate description on the screening procedures. According to this comment, we revised Fig. 1c and provided more detailed description for the screening procedures in the Results (Page 6-8, line 111-182) and Methods sections (Page 27-28, line 811-838). We pointed out the number of candidate genes after each step of screening and supplemented the detailed gene lists for the analyzing processes in Supplementary Table 3. The following revision has been supplemented into the revised manuscript.

Methods (Page 27-28, line 811-838):

Computational Analysis

The present study employed the expression matrix and clinical information of glioma samples from four RNA-seq datasets: CMU RNA-seq cohort (<https://ngdc.cncb.ac.cn/gsa-human/s/3qyKeG8n>), the Chinese Glioma Genome Atlas (CGGA) RNA-seq cohorts, including CGGA325 and CGGA693 cohorts (<http://www.cgga.org.cn>), and the Cancer Genome Atlas RNA-seq cohort (TCGA cohort, <https://tcga-data.nci.nih.gov/tcga/>) (Supplementary Table 1). The ssGSEA R package was used to calculate four metabolic mode scores of each patient in indicated datasets based on corresponding metabolic gene-sets (Glycolysis, oxidative phosphorylation, fatty acid, and glutamine metabolism) downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) (Supplementary Table 2).

GSEA was performed to explore whether the identified sets of genes showed statistical differences between indicated groups. Gene sets were submitted to the DAVID website (<https://david.ncifcrf.gov/>) to perform gene ontology and pathway annotation. GSE131928 GBM, GSE139448 GBM, and GSE171891 glioma datasets were applied for detecting the expression of HEXB in GBM and non-malignant cell populations. There are 8 GBM samples included in GSE117891 glioma dataset, which were subjected for the analysis. GSE5099 dataset was used for detecting the expression of HEXB in different status of macrophages or monocytes. The t-distributed stochastic neighbor embedding (TSNE) analysis with R programming language was employed to access expression patterns of grouped cells in GBM. Limma R package was performed

to identified the DEGs between two different groups ($\text{LogFC} > 0.5$ and $P \text{ value} < 0.05$). Macrophage score reflecting relative macrophage proportions was calculated based on the TIMER2.0 (<https://timer.cistrome.org>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively. Pearson correlation analysis was used to compare the correlation between glycolysis score and macrophage ratio calculated by two algorithms.

Results (Page 6-8, line 111-182):

HEXB is an unfavorable indicator which facilitates the regulation of glioma cell glycolysis associated with TAMs

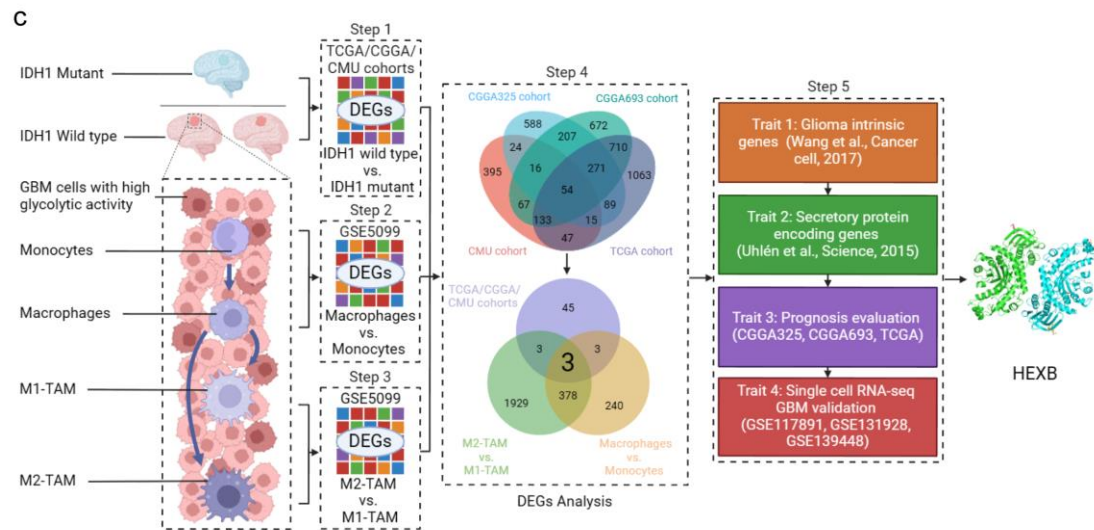
To characterize metabolic promoting signaling in grade IV glioma with divergent IDH1 status, we enrolled grade IV glioma samples from four glioma RNA-seq cohorts (CMU cohort $n = 30$, CGGA325 cohort $n = 139$, CGGA693 cohort $n = 239$, TCGA cohort $n = 148$; Supplementary Table 1) including 556 cases of grade IV gliomas (IDH1 wild type = 453; IDH1 mutant = 103) for analysis. The single-sample gene set enrichment analysis (ssGSEA) algorithms based on representative gene sets of four major metabolic modes, including glycolysis, oxidative phosphorylation, fatty acid and glutamine metabolism (Supplementary Table 2), were used for evaluating the signaling activation of these metabolic modes in grade IV glioma. Then, glycolysis was identified as the metabolic mode with most diverse activation status between IDH1 wild type glioma (Glioblastoma, GBM) and grade IV IDH1 mutant glioma compared to other three metabolic modes (Fig. 1a). We further validated the existed difference in glycolysis levels between grade IV IDH1 mutant and GBM in both male and female patient groups in CGGA325 and CGGA693 cohorts, which indicating the difference in glycolysis levels caused by IDH1 mutation status independent of sex (Supplementary Fig. 1a-d; CMU and TCGA cohorts only include one and two female patients with IDH1 mutation, respectively. Thus, the metabolic capacity of different IDH1 mutation status cannot be statistically determined based on gender). Additionally, we evaluated the sex ratio of glioma patients in the above four glioma datasets and found that the ratio of males to females did not exceed 2:1 (Supplementary Fig. 1e). Meanwhile, we found that sex was not a key factor leading to poor prognosis in glioma patients through univariate Cox (Supplementary Fig. 1f-h) and Kaplan Meier analysis (Supplementary Fig. 1i-k) (CGGA325, CGGA693 and TCGA cohorts; Since the survival information of CMU RNA-seq dataset in Fig. 1a isn't available currently, this analysis didn't include CMU RNA-seq dataset.). Subsequently, we compared the correlation between glycolysis activation and the infiltration of macrophages represented by the above calculated glycolysis score and macrophage scores based on Timer¹² and Xcell algorithms, respectively. We found that the upregulation of glycolysis score was significantly positively correlated with the elevation of macrophage scores. This indicates the increased infiltration of macrophages accompanying with enhanced glycolysis in GBM (Fig. 1b) and suggests the important role of tumor associated macrophages (TAMs) in promoting the glycolytic activity of GBM. Then, we investigated the function and regulatory process of glycolysis in the GBM microenvironment and compared the glycolysis activation status of malignant cells and TAMs in three single-cell RNA-sequencing GBM dataset (scRNA-seq datasets: GSE117891, GSE131928, and GSE139448) with the glycolysis score. We found that GBM cells had a significantly higher glycolysis score than TAMs (Supplementary Fig. 2a-c), which indicated that glycolysis may be more activated in malignant cells than TAMs in GBM.

Next, we sought to screen the crucial regulator in GBM facilitating cancer cell glycolysis associated with TAMs, which is not only expressed by both of GBM cells and TAMs, but also could potentially exhibit its function as secretory protein mediating the crosstalk between GBM cells and TAMs (Fig. 1c). Firstly, we examined differentially expressed genes (DEGs) between GBM and IDH1 mutant grade IV glioma in the above four glioma cohorts, respectively (CMU, CGGA325, CGGA693 and TCGA; Step 1). A total of 54 gene candidates (DEG1, Supplementary Table 3) were obtained from the intersection of these 4 datasets, which were significantly overexpressed in GBM. Secondly, we intersected DEG1 with a characteristic gene list of TAMs summarized from monocyte to macrophage transformation (Step 2) and M2 versus M1 macrophages (Step 3), both of which based on DEG analysis (DEG2, Supplementary Table 3) of a macrophage maturation and polarization dataset (GSE5099). The intersection of DEG1 (n = 54) and DEG2 (n = 381) resulted in 3 genes (SIPA1L1, GUSB and HEXB, Supplementary Table 3) (Step 4). Thirdly, given that the critical role of cancer cell-macrophage crosstalk in driving GBM cells glycolysis addiction and macrophages transformation, we sought to examine the regulator with elevation in both of GBM cells and TAMs and potentially involved in driving glycolysis addiction and tumorigenesis through inter- and intra-cellular mechanisms^{13, 14}. We intersected these 3 genes with a glioma intrinsic gene list (Step 5 Trait 1)¹⁵ and a secretory protein related gene list (Step 5 Trait 2)¹⁶ (Supplementary Table 3). There were two candidates (GUSB and HEXB) meeting this criterion. Then, through prognostic analysis in CGGA and TCGA, we found that HEXB showed a stronger correlation with poor prognosis of GBM and glioma patients compared to CUSB (Step 5 Trait 3, Supplementary Table 4 and Supplementary Fig. 3), and its elevation served as a risk factor for GBM survival independent of sex (Supplementary Fig. 4). Finally, we examined three single-cell RNA-seq GBM datasets, which demonstrated HEXB highly-expressed in both of malignant cells and TAMs in GBM (GSE117891, GSE131928, and GSE139448; Step 5 Trait 4), while its expression was significantly upregulated in GBM compared to grade IV IDH1 mutant glioma (Supplementary Fig. 5). Therefore, we chose HEXB as the research target, which may serve as a potential determinant for TAMs-enhanced malignant cell glycolysis in GBM (Fig. 1c).

References

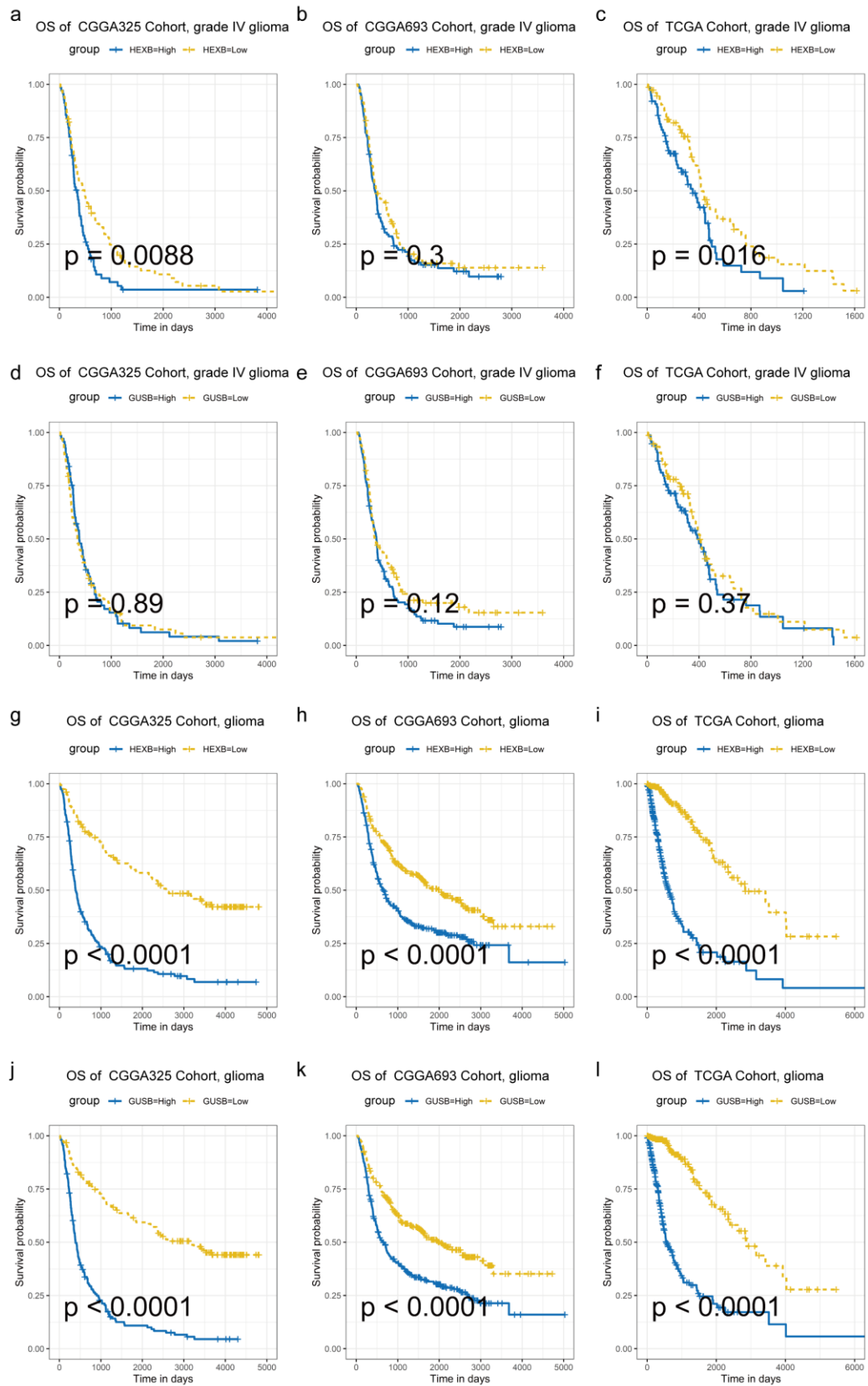
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13. Zhang Y, et al. Macrophage-Associated PGK1 Phosphorylation Promotes Aerobic Glycolysis and Tumorigenesis. *Molecular cell* 71, 201-215.e207 (2018).
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16. Uhlén M, et al. Proteomics. Tissue-based map of the human proteome. *Science* (New York, NY) 347, 1260419 (2015).

Revised Fig. 1c



Revised Fig. 1c: The filtering flow diagram showing the profiling of potential microenvironment related glycolytic targets in indicated GBM cohorts, which identified HEXB as the potential secretory target and malignant and macrophage co-expressed gene in IDH1 wild type glioma.

New Supplementary Fig. 3



Supplementary Fig. 3. The survival analysis in indicated glioma and grade IV glioma according to HEXB and GUSB expression in indicated glioma datasets.

a-f Overall survival curves stratified by HEXB (a-c) and GUSB (d-f) expression in indicated three grade IV glioma cohorts, respectively (Log-rank analysis; a, d: CGGA 325 n = 137, including low group n = 68 and high group n = 69; b, e: CGGA 693 n = 237, including low group n = 118 and high group n = 119; c, f: TCGA n = 152, including low group n = 76 and high group n = 76).

g-l Overall survival curves stratified by HEXB (g-i) and GUSB (j-l) expression in indicated three glioma cohorts (Log-rank; g, j: CGGA 325 n = 313, including low group n = 156 and high group n = 157; h, k: CGGA 693 n = 657, including low group n = 328 and high group n = 329; i, l: TCGA cohort n = 603, including low group n = 301 and high group n = 302).

Reviewer #2 (Remarks to the Author):

In this paper, the authors studied interplay between TAMs and GBM cell glycolysis. They nominated HEXB as a key molecule facilitating cross talk between TAMs and GBM cells through bulk and single cell RNA seq analysis. They showed that HEXB upregulates glycolysis in GBM cells. They also show that inhibition of HEXB leads to survival benefit in vitro and in mice. They propose that HEXB acts through ITGB1-ILK complex and promotes nuclear translocation of YAP-1. This, in turn, activates HIF1alpha which further promotes transcription of HEXB and glycolytic enzymes. They propose that GBM derived HEXB induces chemotaxis of M2 TAMs which secrete more HEXB and this leads to enhanced glycolysis in GBM cells (TAMs-associated glycolysis-promoting network). This article has numerous strengths. The biology described in the paper is novel and of potential therapeutic importance. The authors' experimental design and narrative is very well thought out and logical. The models used are appropriate (both PDXs/GSCs and murine-derived GBMs). They perform rigorous testing of HEXB and its downstream biology in several ways: knockdown, pharmacological inhibition, addition of recombinant protein. All these modulations show consistent results and support their model. Further, they use excellent biochemical studies to identify the specific region and amino acid of HEXB3 that binds to ITGB1. They also intervene directly on the YAP1 with inhibitors and constitutively active models to show that it directly mediates the effects of HEXB. I anticipate this manuscript will have high impact on the field of cancer/immune-metabolism and neuro-oncology. I do have several comments that I hope will help clarify some of the results.

Response: We really appreciate your high evaluation regarding the design and novelty of our work. The following specific suggestions are of great significance and value to improve the quality of our manuscripts.

Major Comments:

1. Line 140- 144. *“According to cross differential analysis of glioma cell intrinsic secretory protein encoding-genes associated with IDH1 status and TAMs polarization in bulk tumor and single cell RNA-sequencing cohorts (Fig.1C), HEXB was identified as a potential determinant for malignant cell glycolysis associated with TAMs (Supplementary Table S2).” The details of nomination of HEXB are unclear. In fig 1C, the source of M1 vs M2 macrophages and monocyte vs macrophage circles in the venn diagram of cross differential analysis are not clear in the legend, text or the supplementary table. It would be helpful if the authors simplified this nomination or explained it in more detail.*

Response: We appreciated this comment and apologized for the inadequate description on the nomination of HEXB in our manuscript. As shown below, we revised Fig. 1c and provided more detailed description for the screening procedures in the Methods (Page 27-28, line 811-838) and Results sections (Page 6-8, line 111-182) of our manuscript. Besides, we supplemented the detailed gene lists for the analyzing processes in Supplementary Table 3.

Methods (Page 27-28, line 811-838):

Computational Analysis

The present study employed the expression matrix and clinical information of glioma samples from four RNA-seq datasets: CMU RNA-seq cohort (<https://ngdc.cncb.ac.cn/gsa-human/s/3qyKeG8n>), the Chinese Glioma Genome Atlas (CGGA) RNA-seq cohorts, including CGGA325 and CGGA693 cohorts (<http://www.cgga.org.cn>), and the Cancer Genome Atlas RNA-seq cohort (TCGA cohort, <https://tcga-data.nci.nih.gov/tcga/>) (Supplementary Table 1). The ssGSEA R package was used to calculate four metabolic mode scores of each patient in indicated datasets based on corresponding metabolic gene-sets (Glycolysis, oxidative phosphorylation, fatty acid, and glutamine metabolism) downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) (Supplementary Table 2).

GSEA was performed to explore whether the identified sets of genes showed statistical differences between indicated groups. Gene sets were submitted to the DAVID website (<https://david.ncifcrf.gov/>) to perform gene ontology and pathway annotation. GSE131928 GBM, GSE139448 GBM, and GSE171891 glioma datasets were applied for detecting the expression of HEXB in GBM and non-malignant cell populations. There are 8 GBM samples included in GSE171891 glioma dataset, which were subjected for the analysis. GSE5099 dataset was used for detecting the expression of HEXB in different status of macrophages or monocytes. The t-distributed stochastic neighbor embedding (TSNE) analysis with R programming language was employed to access expression patterns of grouped cells in GBM. Limma R package was performed to identify the DEGs between two different groups ($\text{LogFC} > 0.5$ and $P \text{ value} < 0.05$). Macrophage score reflecting relative macrophage proportions was calculated based on the TIMER2.0 (<https://timer.cistrome.org>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively. Pearson correlation analysis was used to compare the correlation between glycolysis score and macrophage ratio calculated by two algorithms.

Results (Page 6-8, line 111-182):

HEXB is an unfavorable indicator which facilitates the regulation of glioma cell glycolysis associated with TAMs

To characterize metabolic promoting signaling in grade IV glioma with divergent IDH1 status, we enrolled grade IV glioma samples from four glioma RNA-seq cohorts (CMU cohort $n = 30$, CGGA325 cohort $n = 139$, CGGA693 cohort $n = 239$, TCGA cohort $n = 148$; Supplementary Table 1) including 556 cases of grade IV gliomas (IDH1 wild type = 453; IDH1 mutant = 103) for analysis. The single-sample gene set enrichment analysis (ssGSEA) algorithms based on representative gene sets of four major metabolic modes, including glycolysis, oxidative phosphorylation, fatty acid and glutamine metabolism (Supplementary Table 2), were used for evaluating the signaling activation of these metabolic modes in grade IV glioma. Then, glycolysis was identified as the metabolic mode with most diverse activation status between IDH1 wild type glioma (Glioblastoma, GBM) and grade IV IDH1 mutant glioma compared to other three metabolic modes (Fig. 1a). We further validated the existed difference in glycolysis levels between grade IV IDH1 mutant and GBM in both male and female patient groups in CGGA325 and CGGA693 cohorts, which indicating the difference in glycolysis levels caused by IDH1 mutation status independent of sex (Supplementary Fig. 1a-d; CMU and TCGA cohorts only include one and two female patients with IDH1 mutation, respectively. Thus, the metabolic capacity of different IDH1 mutation status cannot be statistically determined based on gender). Additionally, we evaluated the sex ratio of glioma patients in the above four glioma datasets and found that the ratio of males to females did not exceed 2:1

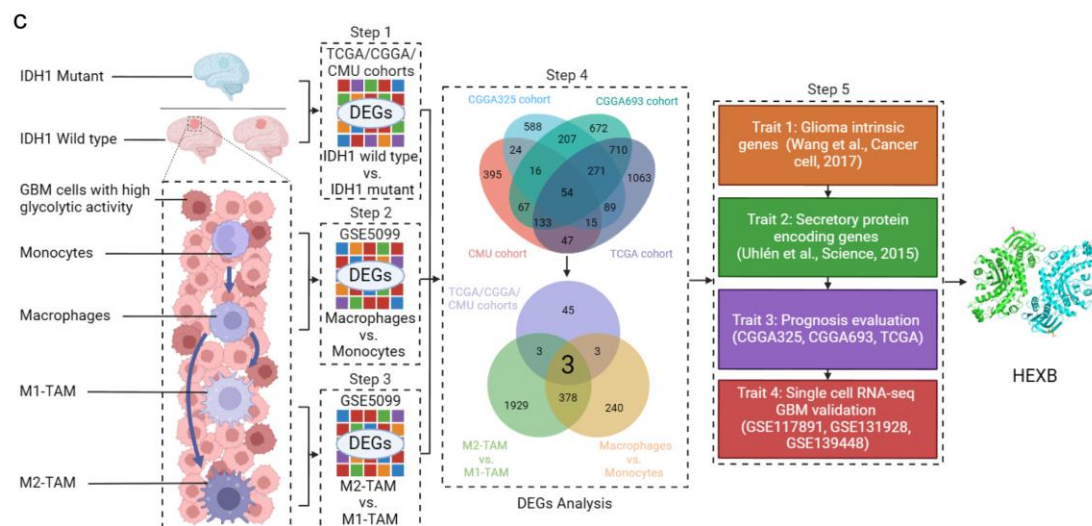
(Supplementary Fig. 1e). Meanwhile, we found that sex was not a key factor leading to poor prognosis in glioma patients through univariate Cox (Supplementary Fig. 1f-h) and Kaplan Meier analysis (Supplementary Fig. 1i-k) (CGGA325, CGGA693 and TCGA cohorts; Since the survival information of CMU RNA-seq dataset in Fig. 1a isn't available currently, this analysis didn't include CMU RNA-seq dataset.). Subsequently, we compared the correlation between glycolysis activation and the infiltration of macrophages represented by the above calculated glycolysis score and macrophage scores based on Timer¹² and Xcell algorithms, respectively. We found that the upregulation of glycolysis score was significantly positively correlated with the elevation of macrophage scores. This indicates the increased infiltration of macrophages accompanying with enhanced glycolysis in GBM (Fig. 1b) and suggests the important role of tumor associated macrophages (TAMs) in promoting the glycolytic activity of GBM. Then, we investigated the function and regulatory process of glycolysis in the GBM microenvironment and compared the glycolysis activation status of malignant cells and TAMs in three single-cell RNA-sequencing GBM dataset (scRNA-seq datasets: GSE117891, GSE131928, and GSE139448) with the glycolysis score. We found that GBM cells had a significantly higher glycolysis score than TAMs (Supplementary Fig. 2a-c), which indicated that glycolysis may be more activated in malignant cells than TAMs in GBM.

Next, we sought to screen the crucial regulator in GBM facilitating cancer cell glycolysis associated with TAMs, which is not only expressed by both of GBM cells and TAMs, but also could potentially exhibit its function as secretory protein mediating the crosstalk between GBM cells and TAMs (Fig. 1c). Firstly, we examined differentially expressed genes (DEGs) between GBM and IDH1 mutant grade IV glioma in the above four glioma cohorts, respectively (CMU, CGGA325, CGGA693 and TCGA; Step 1). A total of 54 gene candidates (DEG1, Supplementary Table 3) were obtained from the intersection of these 4 datasets, which were significantly overexpressed in GBM. Secondly, we intersected DEG1 with a characteristic gene list of TAMs summarized from monocyte to macrophage transformation (Step 2) and M2 versus M1 macrophages (Step 3), both of which based on DEG analysis (DEG2, Supplementary Table 3) of a macrophage maturation and polarization dataset (GSE5099). The intersection of DEG1 (n = 54) and DEG2 (n = 381) resulted in 3 genes (SIPA1L1, GUSB and HEXB, Supplementary Table 3) (Step 4). Thirdly, given that the critical role of cancer cell-macrophage crosstalk in driving GBM cells glycolysis addiction and macrophages transformation, we sought to examine the regulator with elevation in both of GBM cells and TAMs and potentially involved in driving glycolysis addiction and tumorigenesis through inter- and intra-cellular mechanisms^{13, 14}. We intersected these 3 genes with a glioma intrinsic gene list (Step 5 Trait 1)¹⁵ and a secretory protein related gene list (Step 5 Trait 2)¹⁶ (Supplementary Table 3). There were two candidates (GUSB and HEXB) meeting this criterion. Then, through prognostic analysis in CGGA and TCGA, we found that HEXB showed a stronger correlation with poor prognosis of GBM and glioma patients compared to CUSB (Step 5 Trait 3, Supplementary Table 4 and Supplementary Fig. 3), and its elevation served as a risk factor for GBM survival independent of sex (Supplementary Fig. 4). Finally, we examined three single-cell RNA-seq GBM datasets, which demonstrated HEXB highly-expressed in both of malignant cells and TAMs in GBM (GSE117891, GSE131928, and GSE139448; Step 5 Trait 4), while its expression was significantly upregulated in GBM compared to grade IV IDH1 mutant glioma (Supplementary Fig. 5). Therefore, we chose HEXB as the research target, which may serve as a potential determinant for TAMs-enhanced malignant cell glycolysis in GBM (Fig. 1c).

References

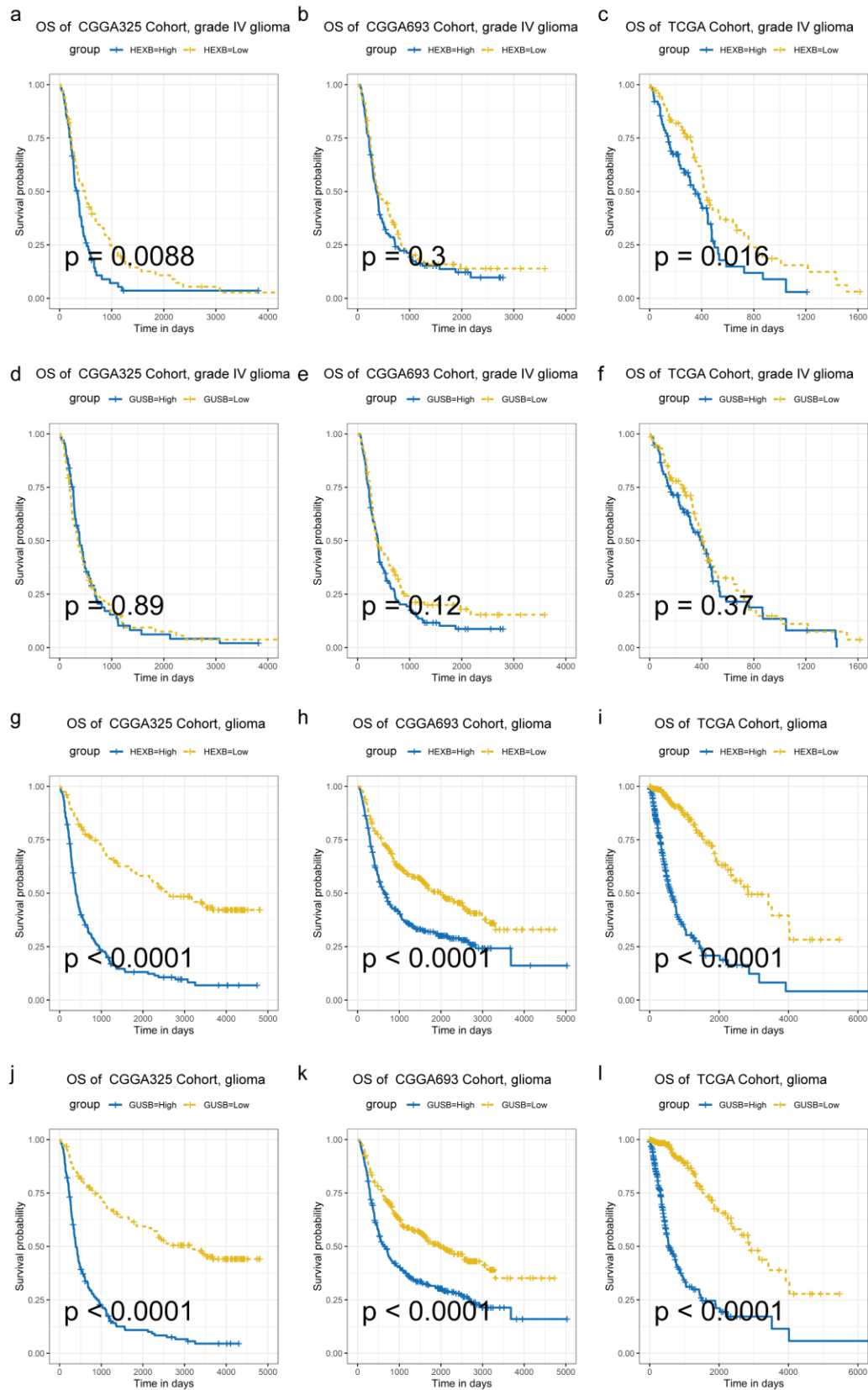
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Revised Fig. 1c



Revised Fig. 1c: The filtering flow diagram showing the profiling of potential microenvironment related glycolytic targets in indicated GBM cohorts, which identified HEXB as the potential secretory target and malignant and macrophage co-expressed gene in IDH1 wild type glioma.

New Supplementary Fig. 3



Supplementary Fig. 3. The survival analysis in indicated glioma and grade IV glioma according to HEXB and GUSB expression in indicated glioma datasets.

a-f Overall survival curves stratified by HEXB (a-c) and GUSB (d-f) expression in indicated three grade IV glioma cohorts, respectively (Log-rank analysis; a, d: CGGA 325 n = 137, including low group n = 68 and high group n = 69; b, e: CGGA 693 n = 237, including low group n = 118 and high group n = 119; c, f: TCGA n = 152, including low group n = 76 and high group n = 76).

g-l Overall survival curves stratified by HEXB (g-i) and GUSB (j-l) expression in indicated three glioma cohorts (Log-rank; g, j: CGGA 325 n = 313, including low group n = 156 and high group n = 157; h, k: CGGA 693 n = 657, including low group n = 328 and high group n = 329; i, l: TCGA cohort n = 603, including low group n = 301 and high group n = 302).

2. Line 350-356: The co-injection model was not described in the methods, nor was any reference cited in the main text. Please provide a reference showing that the macrophages co-injected along with GBM cells survive and can therefore mediate the proposed effects on tumor growth. Alternatively, please show any IHC images that show higher abundance of M2 macrophages in the co-injected tumors when compared to GSC injection only tumors. Or if any labelling of macrophages was performed prior to injection, please show the labelled macrophages in the tumor.

Response: We thank the reviewer for this comment. According to this suggestion, we have supplemented the description of co-injection model method into the methodology part and cited relevant references as follows (Page 23, line 664-667):

In vivo GBM cells and macrophages co-implantation experiments were performed as previously described^{8, 57, 58, 59, 60, 61}. Briefly, a 3 μ l cell suspension (2×10^5 indicated GBM cells with or without 1×10^4 THP-1 derived macrophages) were intracranially implanted into BALB/c nude mice.

Previously, several research teams have employed the co-injection model of glioma cells and macrophages in their studies and confirmed that the macrophages co-injected along with GBM cells could survive and further mediate the proposed effects on the intracranial orthotopic transplantation of GSC or GBM cell growth^{8, 57, 58, 59, 60, 61}. Moreover, we applied an anti-human CD163 antibody (GB113152, Servicebio) to perform IHC staining of brain tumor sections derived from GSC1 and THP-1 co-injection experiments (Revised Fig. 6k) and confirmed the survival of THP-1 derived macrophages co-injected along with GBM cells (Reviewer only Fig. 2, the human CD163 positive staining cells shown below).

References

8. Wang W, et al. Identification of hypoxic macrophages in glioblastoma with therapeutic potential for vasculature normalization. *Cancer cell* 42, 815-832.e812 (2024).
57. Zhou W, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nature cell biology* 17, 170-182 (2015).
58. Mei Y, et al. Siglec-9 acts as an immune-checkpoint molecule on macrophages in glioblastoma, restricting T-cell priming and immunotherapy response. *Nature cancer* 4, 1273-1291 (2023).
59. Shi Y, et al. Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth. *Nature communications* 8, 15080 (2017).
60. Zhao L, et al. Lymphatic endothelial-like cells promote glioblastoma stem cell growth

through cytokine-driven cholesterol metabolism. Nature cancer 5, 147-166 (2024).

61. Liu X, et al. CD16(+) fibroblasts foster a trastuzumab-refractory microenvironment that is reversed by VAV2 inhibition. Cancer cell 40, 1341-1357.e1313 (2022).

Fig. 6k

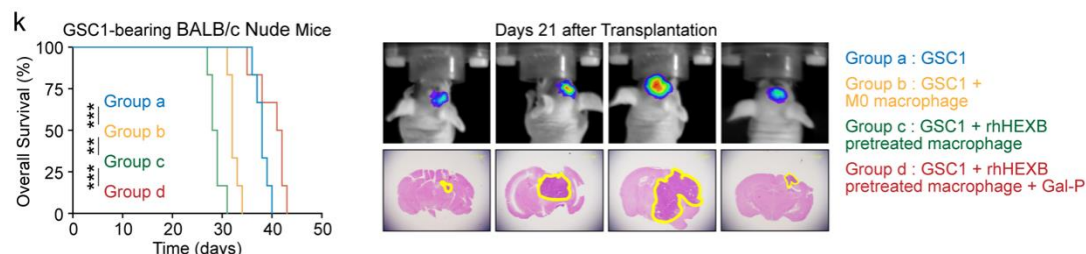
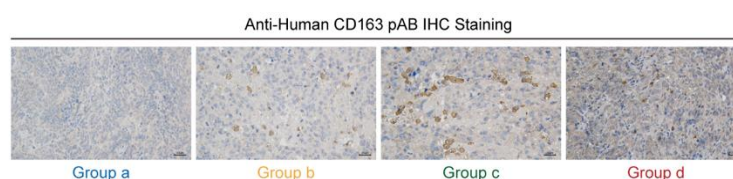


Fig. 6k: Survival curves (Left) and representative tumor images (Right: in vivo bioluminescence (Upper) and H&E images (Lower) at 21 days after the mice intracranial co-implantation of GSC1 and THP-1 derived macrophages) of indicated GSC1 orthotopic models (Group a: GSC1; Group b: GSC1+M0 macrophage; Group c: GSC1+rhHEXB pretreated macrophage; Group d: GSC1+rhHEXB pretreated macrophage with administration of Gal-P).

Reviewer only Fig. 2



Reviewer only Fig. 2: Representative IHC staining images of human CD163 in brain tumor sections derived from GSC1 and THP-1 co-injection experiments in Fig. 6k (Group a: GSC1; Group b: GSC1+M0 macrophage; Group c: GSC1+rhHEXB pretreated macrophage; Group d: GSC1+rhHEXB pretreated macrophage with administration of Gal-P).

Minor comments:

1. Line 279: Please provide reference for the statement “It was reported that YAP1 could bind with HIF1a to maintain its stability and further activate transcription of downstream oncogenic glycolytic genes (like PKM2) to enhance glycolytic activity.”

Response: We thank for this comment. The reference (*J Exp Clin Cancer Res.* 2018. **PMID: 30180863**) was supplemented into the revised manuscript accordingly in Page 13, line 370.

2. Line 330: Fig 6B shows positive correlation and co-localization of HEXB with M2 macrophage marker, CD163. Was CD80 or CD86, which are markers for M1 macrophages, also tested for colocalization with HEXB to show that it does not show correlation? The details of the Sleeping beauty-GSC model are not mentioned in the manuscript or supplementary material. There is also no reference to any previous publications by the authors using this model. Please provide a reference or the details of how the sleeping beauty model was developed.

Response: Thank you for this precious comment, which further enhances the logic and preciseness of our work. According to this suggestion, we have improved the immunohistochemical staining of tissue samples in Fig. 6b. We further performed the IHC staining of M1 macrophage marker CD86 and found that its expression in GBM was low and had no positive correlation with the expression of HEXB (New Supplementary Fig. 15f). Besides, we observed that there was almost no co-localization between HEXB and CD86 by tissue polychromatic immunofluorescence staining (Revised Fig. 1f). We have revised Results section accordingly (Page 15-16, line 449-451).

We apologized that we didn't give enough details of Sleeping Beauty (SB)-GSC model. Our group have established a spontaneous glioma model using Sleeping Beauty transposon system skillfully as previously reported^{55, 56}. SB is a two-part system consisting of transposon DNA and transposase. SB transposase could cut off the transposon DNA and paste it into the TA dinucleotide of the target genome. We separated the transposase and transposon on two different plasmids. The transposon plasmid with oncogenic genes (SV40-LgT and NRAS-G12V) was mixed with the plasmid encoding transposase and injected into the brain of fetal mouse (within 24 hours after born) to induce spontaneous glioma. According to this comment, we made the following revision to the manuscript to provide the details of the establishing method for the SB mGSC model in the Methods section, and cited SB relevant articles previously published as reference 55 and 56 in the revised manuscript.

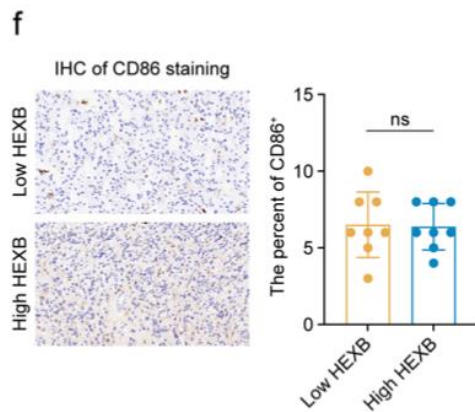
Methods (Page 22, line 636-647)

mGSCs were extracted from murine spontaneous GBM established by sleeping beauty (SB) transposon method described before^{55, 56}. Briefly, SB was a two-part system consisting of transposon DNA and transposase. SB transposase could cut off the transposon DNA and paste it into the TA dinucleotide of the target genome. We separated the transposase and transposon on two different plasmids. The transposon plasmid with oncogenic genes (SV40-LgT and NRAS-G12V) was mixed with the plasmid encoding transposase and injected into the brain of fetal mouse (within 24 h after born) to induce spontaneous glioma. After tumor formation, the tumor specimen in the brain of mice were collected and cut into small pieces, digested into single cells with Accutase. After red blood cells were lysed by Red Blood Cell Lysis Buffer, cell suspensions were passed through a 70 μ m stainless steel mesh and re-cultured in the serum-free stem cell medium.

References

55. Liu T, et al. Ferroptosis, as the most enriched programmed cell death process in glioma, induces immunosuppression and immunotherapy resistance. *Neuro-oncology* 24, 1113-1125 (2022).
56. Wiesner SM, et al. De novo induction of genetically engineered brain tumors in mice using plasmid DNA. *Cancer research* 69, 431-439 (2009).

New Supplementary Fig. 15f, related to Fig. 6



New Supplementary Fig. 15f: Representative IHC images (left) and positive cell ratio analysis (right) of CD86⁺ staining in GBM samples with low and high HEXB expression (n = 16, including low HEXB n = 8, high HEXB n = 8; t-test).

3. Typographical errors:

There are a few typographical errors intermittently in the manuscript. Example:

Line 104: Sing-cell instead of single-cell

Line 191: Sleep beauty instead of sleeping beauty.

Response: Thank you and apologize for these typographical errors in our manuscript. We have revised the text in the manuscript accordingly.

Reviewer #3 (Remarks to the Author):

Zhu et al compared gene expression in IDH-wildtype and mutant grade 4 gliomas using public RNA-seq data. They identified glycolysis as the main metabolic difference between the two indications, and a positive correlation with TAM content. They identify HEXB as correlating with this signature and note its expression in GBM cells and in macrophages. They found that perturbation of HEXB alters survival in vivo and that HEXB enhances glycolysis by activating YAP1 nuclear translocation. The study is rigorously done and the conclusions are largely supported by the data. I would ask the authors to consider the following points.

Response: We deeply appreciate your thoughtful and positive comments. These comments are of great significance and value to improve the quality of our manuscripts. The following responses are the replies to these comments.

1. The authors cite prior reports of TAMs constituting 30%-50% of the cellular tumor. However, more recent assessments would place this figure closer to 10%-15% in human GBMs.

Response: We appreciate this comment. According to this suggestion, we reviewed and retrieved the latest evaluation reports on the constitution ratio of TAMs in GBM. It is reported that TAMs account for about 10% - 30% of GBM tumor mass^{7,8}. We have revised the manuscript accordingly (Page 4, line 80).

References

7. Friebel E, et al. Single-Cell Mapping of Human Brain Cancer Reveals Tumor-Specific Instruction of Tissue-Invasive Leukocytes. *Cell* 181, 1626-1642.e1620 (2020).
8. Wang W, et al. Identification of hypoxic macrophages in glioblastoma with therapeutic potential for vasculature normalization. *Cancer cell* 42, 815-832.e812 (2024).

2. The authors mention they used “well-recognized” macrophage and glycolysis scores as input to the Timer method. Please specify the source and justification for these scores in the supplement.

Response: We appreciate the reviewer for this suggestion. The glycolysis score was calculated by ssGSEA algorithm based on glycolysis gene-set downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>). The detailed gene list is provided in new Supplementary Table 2. Moreover, the macrophage score was estimated based on TIMER2.0 (<https://timer.cistrome.org>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithms. We have supplemented the following description of establishing “*macrophage and glycolysis scores*” in methods sections shown below in **bold** to provide the information of the source and justification of macrophage and glycolysis scores (Macrophage scores: Page 28 line 834-836; Glycolysis score: Page 27, line 817-822).

Methods (Page 27-28, line 811-838):

Computational Analysis

The present study employed the expression matrix and clinical information of glioma samples from four RNA-seq datasets: CMU RNA-seq cohort (<https://ngdc.cncb.ac.cn/gsa-human/s/3qyKeG8n>), the Chinese Glioma Genome Atlas (CGGA) RNA-seq cohorts, including CGGA325 and CGGA693

cohort (<http://www.cgga.org.cn>), and the Cancer Genome Atlas RNA-seq cohort (TCGA cohort, <https://tcga-data.nci.nih.gov/tcga/>) (Supplementary Table 1). **The ssGSEA R package was used to calculate four metabolic mode scores of each patient in indicated datasets based on corresponding metabolic gene-sets (Glycolysis, oxidative phosphorylation, fatty acid, and glutamine metabolism) downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) (Supplementary Table 2).**

GSEA was performed to explore whether the identified sets of genes showed statistical differences between indicated groups. Gene sets were submitted to the DAVID website (<https://david.ncifcrf.gov/>) to perform gene ontology and pathway annotation. GSE131928 GBM, GSE139448 GBM, and GSE171891 glioma datasets were applied for detecting the expression of HEXB in GBM and non-malignant cell populations. There are 8 GBM samples included in GSE117891 glioma dataset, which were subjected for the analysis. GSE5099 dataset was used for detecting the expression of HEXB in different status of macrophages or monocytes. The t-distributed stochastic neighbor embedding (TSNE) analysis with R programming language was employed to access expression patterns of grouped cells in GBM. Limma R package was performed to identified the DEGs between two different groups (LogFC > 1 and P value < 0.05). **Macrophage score reflecting relative macrophage proportions was calculated based on the TIMER2.0 (<https://timer.cistrome.org>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively.** Pearson correlation analysis was used to compare the correlation between glycolysis score and macrophage ratio calculated by two algorithms.

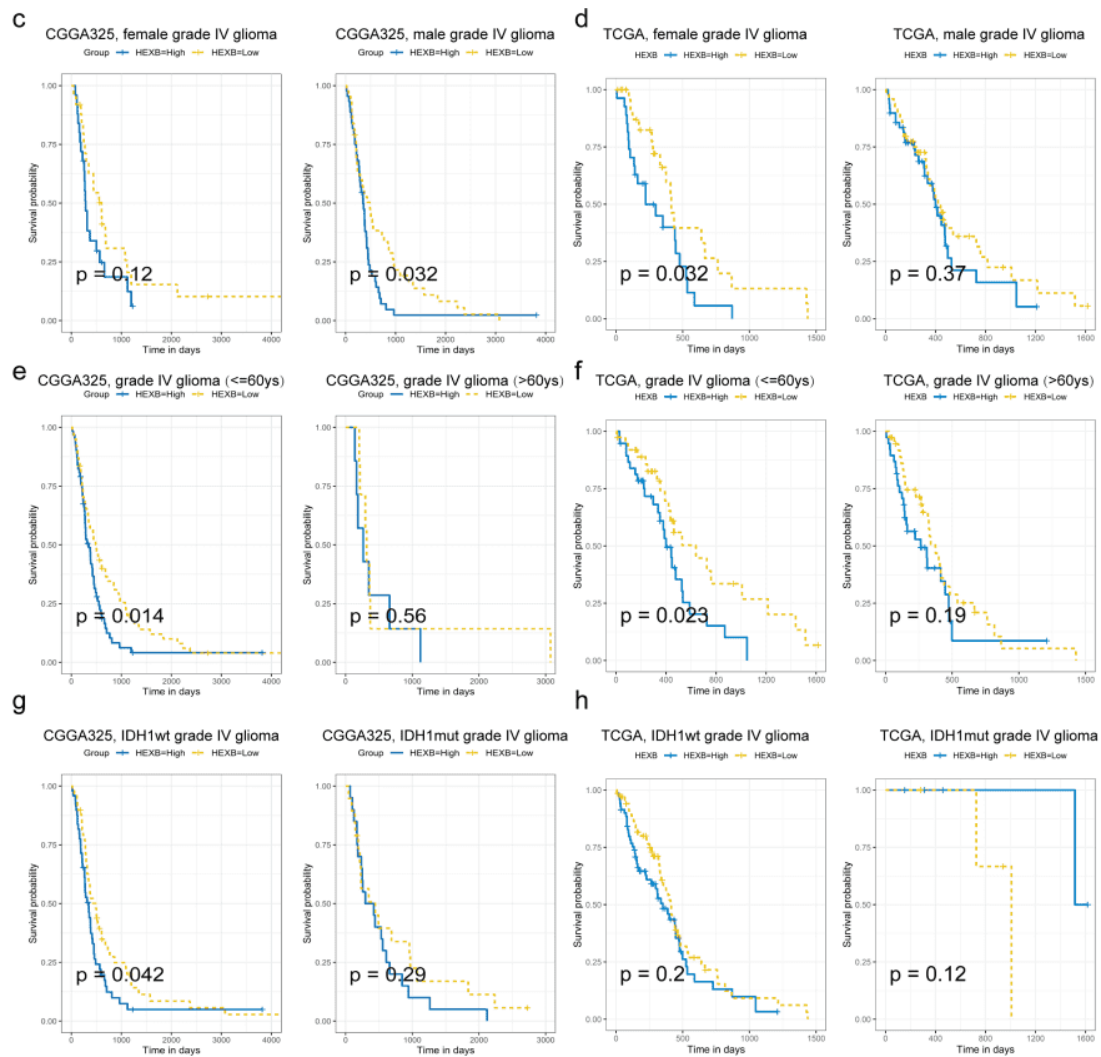
Reference

12. Li T, et al. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic acids research* 48, W509-w514 (2020).

3. The survival analysis of supplementary fig 1k-l or figure 7E-F do not appear to be controlled for IDH mutation status, age or sex.

Response: We thank the reviewer for this comment. The survival analysis in previous supplementary Fig. 1k, l and Fig. 7e, f was performed based on IDH1 status. Therefore, we further improved the survival analysis with grouping according to IDH mutation status, age and sex (New Supplementary Fig. 4c-h and New Supplementary Fig. 18a-e), and have revised the corresponding text in the manuscript (Page 7, line 175-176).

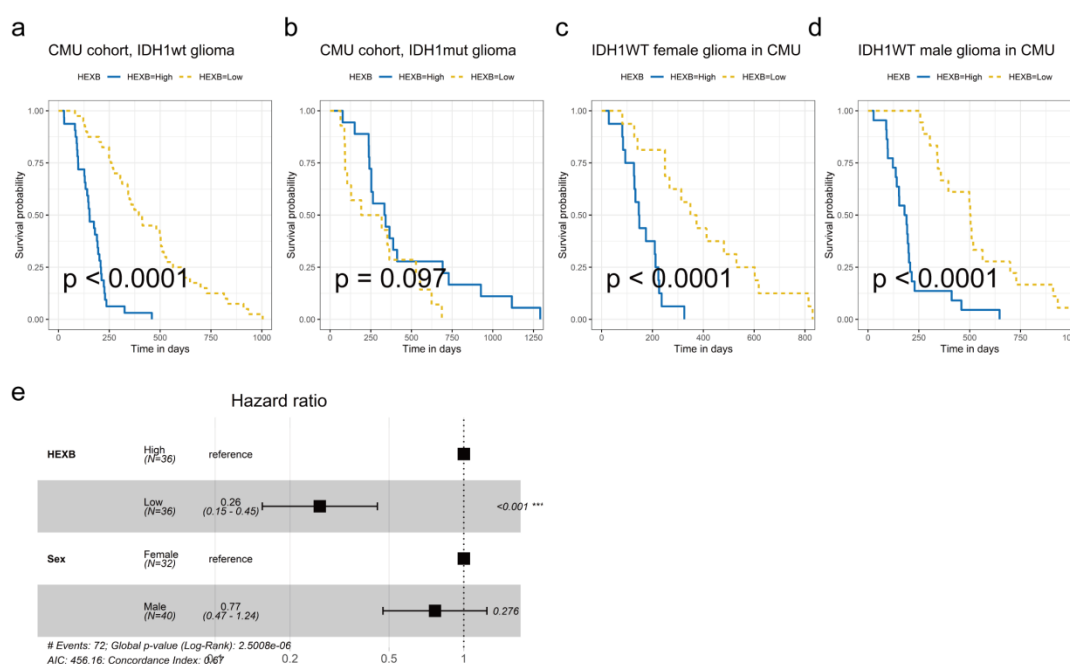
New Supplementary Fig. 4c-h, related to previous supplementary Fig. 1k, l



New Supplementary Fig. 4

c-h The survival analysis of HEXB with high and low groups according to sex (c, d), ages (e, f) and IDH1 status (g, h) (CGGA325 cohort: male n = 87, low group n = 43, high n = 44; female n = 50, low group n = 25, high group n = 25; age ≤ 60 years n = 123, low group n = 61, high group n = 62; age > 60 n = 14, low group n = 7, high group n = 7; IDH1 wild type n = 98, low group n = 49, high group n = 49; IDH1 mutant n = 39, low group n = 19, high group n = 20; TCGA cohort: male n = 98, low group n = 49, high n = 49; female n = 54, low group n = 27, high group n = 27; age ≤ 60 years n = 76, low group n = 38, high group n = 38; age > 60 n = 76, low group n = 38, high group n = 38; IDH1 wild type n = 139, low group n = 69, high group n = 70; IDH1 mutant n = 9, low group n = 4, high group n = 5).

New Supplementary Fig. 18 a-e, related to previous Fig. 7 e, f



New Supplementary Fig. 18c-e, related to Fig. 7 e-f

a, b Overall survival curves stratified by HEXB expression in CMU IDH1 wild type and mutant glioma samples (IDH1 wild type, $n = 72$ (high group, $n = 32$; low group, $n = 40$); IDH1 mutant, $n = 32$ (high group, $n = 18$; low group, $n = 14$); Log-rank).

c, d Overall survival curves of IDH wild type glioma patient stratified by HEXB in CMU glioma dataset according to sex (female, $n = 32$ (high group, $n = 16$; low group, $n = 16$); male, $n = 40$ (high group, $n = 22$; low group, $n = 18$); Log-rank).

e Multivariate Cox analysis of HEXB expression and sex in CMU glioma dataset ($n = 72$) disclosing HEXB as a prognostic factor independent of sex in IDH1 wild type glioma.

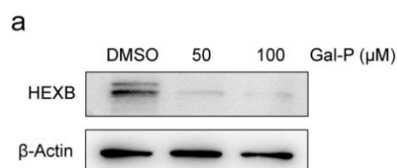
4. Many of the results are obtained from the use of GAL-PUGNAc which is an OGA inhibitor. Can the authors provide evidence that this compound directly targets HEXB?

Response: We thank the reviewer for this thoughtful comment. Firstly, there are direct evidences from a published report to support GAL-PUGNAc (Gal-P) as a specific inhibitor of HEXA and HEXB, and its inhibitory effect on HEXB is more specific²⁰. Secondly, we have tested the inhibition efficiency of Gal-P on HEXB expression in GSCs, which demonstrated that 24h treatment of 50 and 100 μM significantly reduced HEXB expression in these cells (Previous Supplementary Fig. 6a upper panel). Moreover, we reconstructed the structure of Gal-P through structural bioinformatics analysis, and found that it could dock well with HEXB protein (3D molecular simulation docking between HEXB and Gal-P based on Swiss-model and HDOCK software). This also supports the direct targeting of Gal-P at HEXB (New Supplementary Fig. 8e).

Reference

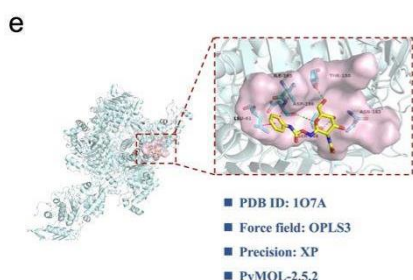
20. Stubbs K, Macauley M, Vocadlo D. A selective inhibitor Gal-PUGNAc of human lysosomal beta-hexosaminidases modulates levels of the ganglioside GM2 in neuroblastoma cells. *Angewandte Chemie (International ed in English)* 48, 1300-1303 (2009).

Previous Supplementary Fig. 6a upper panel (Current Supplementary Fig.14a, upper panel)



Supplementary Fig.14a: Western blotting analysis of HEXB in GSC1 treated with indicated concentration of Gal-P (Upper panel, 24 h).

New Supplementary Fig. 8e, related to Fig. 2



New Supplementary Fig. 8e: 3D molecular simulation docking between HEXB and Gal-P based on Swiss-model and HDOCK software.

5. The authors show that genetic perturbation of HEXB modulates survival in xenografts and that GAL-PUGNAc treatments prolongs survival in xenografts and in syngeneic SB-based models. Does genetic inhibition of HEXB in the syngeneic SB model enhance survival? Over-expression? Does this depend on adaptive and/or innate immunity? The studies in immunocompetent mice are all done with Gal-P but not with genetic manipulation and as such it is not clear if modulation of HEXB is causing the differences in and to what extent this depends on immune infiltrates.

Response: We appreciate the reviewer for this thoughtful comment. Because this study focuses on the exocrine function of HEXB, which refers to the effect and mechanism in the extracellular and cell membrane, endogenous HEXB overexpression has not been employed in current study, so as not to be confused with its intracellular mechanism. According to this suggestion, we performed lenti-viral mediated Hexb knockdown in SB mGSCs, which was generated from a murine spontaneous glioma model created by SB system as previously reported^{55,56}. Then, SB mGSCs with Hexb knockdown or negative control were intracerebrally implanted in C57BL6 mice. Survival analysis revealed that Hexb knockdown (New Supplementary Fig. 16a) significantly prolonged mice survival (New Supplementary Fig. 16b). Moreover, immunohistochemical staining showed that the ratio of CD163 and IBA1 positive cells were significantly reduced by genetic inhibition of Hexb in SB mGSCs, which indicates macrophage infiltration, especially M2 macrophage infiltration, was restrained with Hexb knockdown in SB mGSCs (New Supplementary Fig. 16c and d). These results support the promoting role of HEXB in the progression of GBM through regulating the chemotaxis of macrophages and M2 polarization in immunocompetent mice. Meanwhile, we established murine spontaneous GBM model with Hexb knockdown or negative control by SB transposon system, and isolated SB mGSCs from these models (SB-shNC cells and SB-shHexb cells). Then, we implanted these cells into mice brain (5×10^4 SB-shNC or SB-shHexb intracranially

transplantation, respectively), and found that the survival time of mice transplanted by SB-shHexb cells was significantly prolonged than the mice implanted with SB-shNC cells, and no tumorigenesis was found by H&E staining (Reviewer only Fig. 3). This also support the promoting role of HEXB in the initiation and progression of GBM.

Considering the critical role of TAMs in regulating adaptive immunity in TME (*Nat Immunol.* 2022. **PMID: 35879449**) and the observation of Hexb inhibition on reducing TAMs recruitment, it could be assumed that HEXB promote GBM progression through facilitating the crosstalk between TAMs and GBM cells, which indicates HEXB functions partially depending on adaptive immunity regulation in TME. Combining with the previous data of HEXB ablation in immune incompetent mice to reduce tumorigenicity of GSCs (previous Fig. 1g and 6k), we consider that HEXB exhibits its roles in modulating GBM glycolysis and tumorigenesis through cancer cell-macrophage co-dependency mechanisms and revised the manuscript as follows:

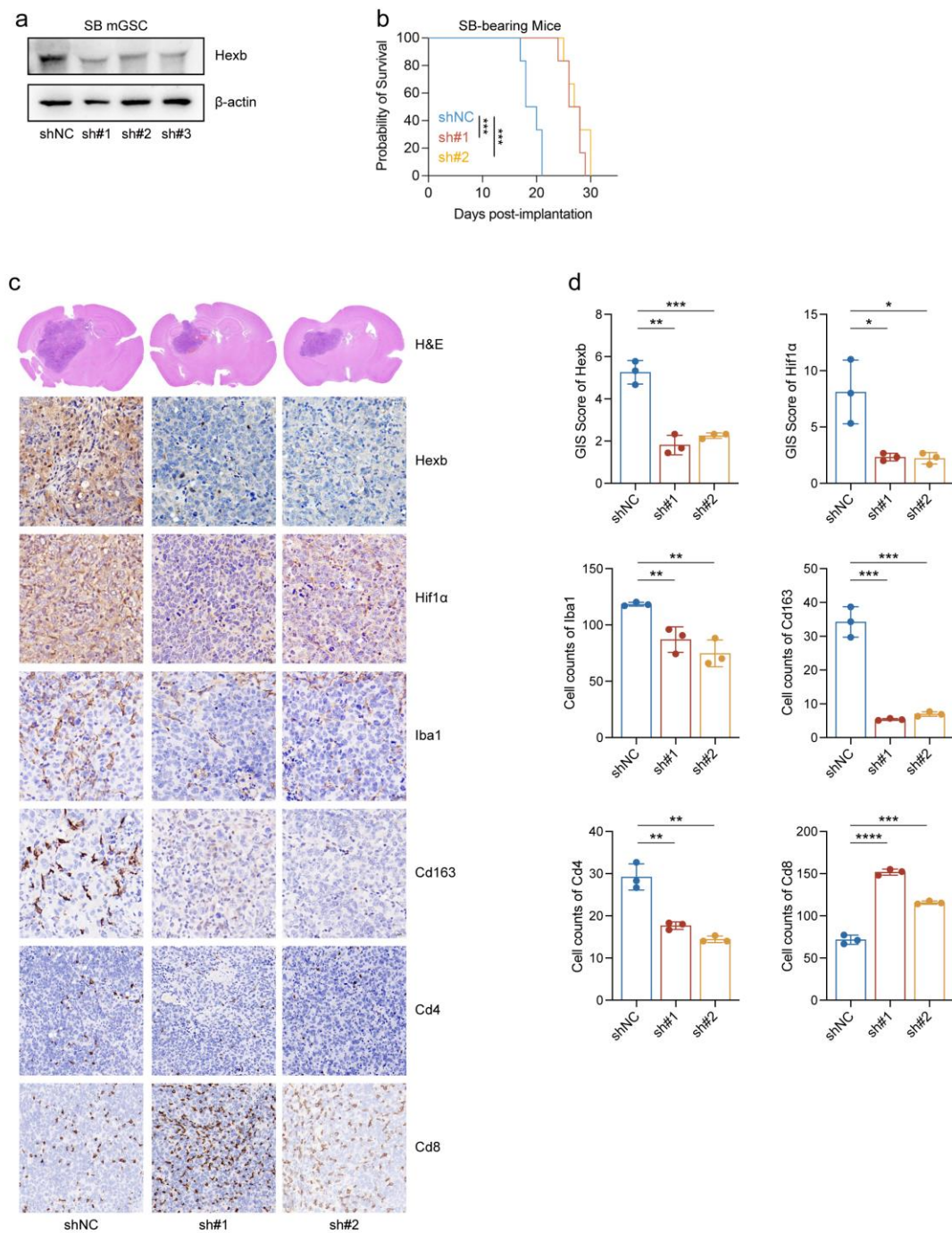
Result section (Page 17, line 490-500)

Then, we performed lenti-viral mediated Hexb knockdown in mGSCs. mGSCs with Hexb knockdown or negative control were intracerebrally implanted in C57BL6 mice. Survival analysis revealed that Hexb knockdown (Supplementary Fig. 16a) significantly prolonged mice survival (Supplementary Fig. 16b). Immunohistochemical staining also showed that the ratio of CD163 and IBA1 positive cells in intracranial tumor samples derived from mGSCs were significantly reduced by genetic inhibition of Hexb in these cells, which indicates macrophage infiltration, especially M2 macrophage infiltration, was restrained with Hexb knockdown in mGSCs (Supplementary Fig. 16c and d). Together, these results support the promoting role of HEXB in the progression of GBM through regulating the chemotaxis of macrophages and M2 polarization in immunocompetent mice.

References

55. Liu T, et al. Ferroptosis, as the most enriched programmed cell death process in glioma, induces immunosuppression and immunotherapy resistance. *Neuro-oncology* 24, 1113-1125 (2022).
 56. Wiesner SM, et al. De novo induction of genetically engineered brain tumors in mice using plasmid DNA. *Cancer research* 69, 431-439 (2009).
- PMID: 35879449.** Christofides A, Strauss L, Yeo A, Cao C, Charest A, Boussiotis VA. The complex role of tumor-infiltrating macrophages. *Nature immunology* 23, 1148-1156 (2022).

New Supplementary Fig. 16, related to Fig. 6



New Supplementary Fig. 16, related to Fig. 6

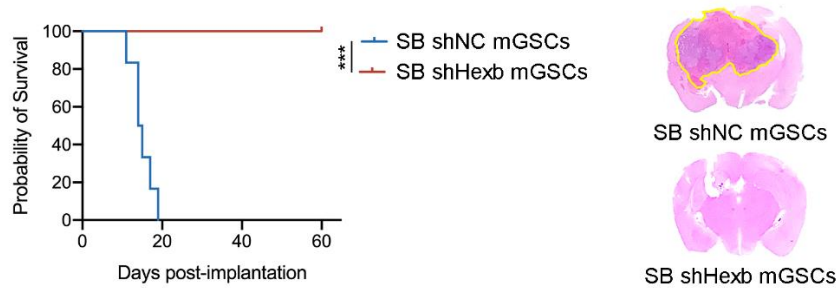
a Western blotting analysis of Hexb expression in indicated SB mGSCs after Hexb-knockdown lentiviruses transfections.

b The survival analysis of tumor-bearing mice after intracranial implantation of SB mGSCs transduced with indicated Hexb knockdown or negative control vector (Log-rank test, $n = 6$).

c Representative H&E (day 16) and IHC staining images (Cd4, Cd8, Hexb, Hif1 α , Iba1, and Cd163) of mice tumor section derived from intracranial implantation of SB mGSCs transduced with indicated Hexb knockdown or negative control vector.

d The IHC staining analysis of indicated markers (Hexb, Hif1 α , Cd4, Cd8, Iba1 and Cd163) in mice tumor section derived from intracranial implantation of SB mGSCs transduced with indicated Hexb knockdown or negative control vector (n = 3, t-test).

Reviewer only Fig. 3



Reviewer only Fig. 3

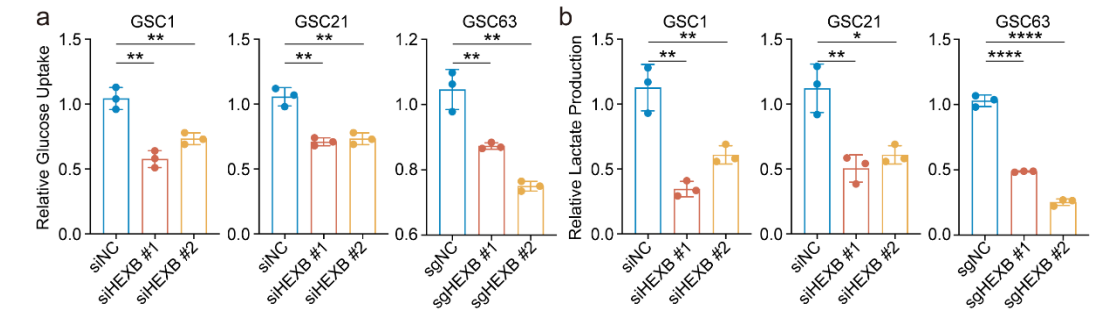
Left The survival analysis of mice intracranial transplanted with Sleeping Beauty (SB) shNC or SB shHexb mGSCs, respectively (SB shNC cells and SB shHexb cells were isolated from murine spontaneous GBM model established by sleeping beauty control or Hexb knockdown transposon plasmid, respectively; n = 6, log rank).

Right Representative H&E staining images of mice brain sections transplanted with mGSCs derived from mouse GBM established by SB shNC (Upper) or SB shHexb (Lower) transposon vectors, respectively.

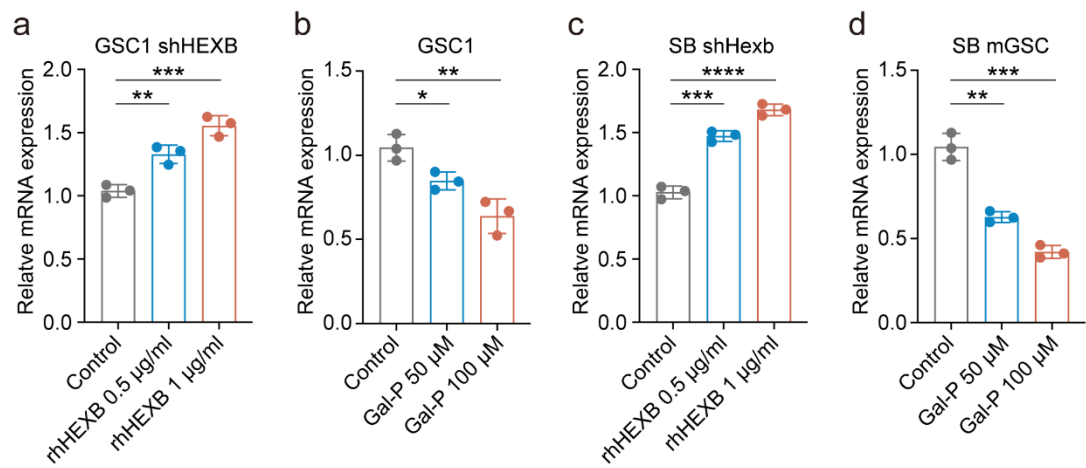
6. In figure 2A-B the authors note statistical significance, but there are no replicates of the non-targeting control, similarly for figure 5A-D.

Response: We thank the reviewer for this comment. Indeed, there are replicates in non-targeted control group. During the preparation of the figures, we homogenized the NC group, resulting in this misunderstanding. We apologize for this negligence, and we have revised the manuscript accordingly.

Revised Fig. 2a and b



Revised Fig. 5a-d



Reviewer #4 (Remarks to the Author):

The manuscript “HEXB-driven cancer cell-macrophage co-dependency promotes glycolysis addiction and tumorigenesis in glioblastoma” identifies HEXB as a regulator for glycolysis in IDH1 wildtype glioblastoma, and reports HEXB impacts TAMs recruitment and polarization. A strength of this manuscript is that it investigates both the molecular mechanism of HEXB’s action and the therapeutic potential of targeting HEXB, providing a large amount of data. However, a general issue is that specific methods and exact controls for many experiments are not clearly described, making it difficult to review the scientific merits of these experiments. The writing of the manuscript needs to be improved as well. Some specific comments are below.

Response: Thank you for the pertinent and objective comments, which is constructive to present work. The specific responses to each comment are listed below.

1. It is unclear what exact result is being analyzed in Figure 1A-B and how the gene-sets are defined. It appears to be RNAseq of tumor samples, but unclear whether it is bulk RNA-seq of single cell RNAseq, and how is the data normalized.

Response: We thank you for your precious comment and apologize for the insufficient description of result and the gene-sets employed for the screening in Fig. 1a and b. The data of Fig. 1a and b was obtained from the analysis with bulk RNA-seq data of clinical grade IV glioma samples (CMU cohorts, n = 30) and public bulk glioma RNA-seq datasets (CGGA325 cohort, n = 139; CGGA693 cohort, n = 239; TCGA cohort, n = 148), which totally included 556 gliomas (IDH1 wild type n = 453; IDH1 mutant n = 103). RNA-seq mapping and quantification were processed by using STAR (v2.5.2b) and RSEM (v1.2.31) software. Briefly, the reads were aligned to the human genome reference (GENCODE v19, hg19) with STAR, and then sequencing read counts for each GENCODE gene were calculated using RSEM. The expression levels of different samples were merged into a FPKM (fragments per kilobase transcriptome per million fragments) matrix. Meanwhile, the metabolic scores mentioned in Fig. 1a and b were estimated by ssGSEA algorithm based on metabolic gene-sets (Glycolysis, oxidative phosphorylation, fatty acid, and glutamine metabolism) downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>, Supplementary Table 2). Macrophage score mentioned in Fig. 1b were calculated based on the TIMER2.0 (<https://timer.cistrome.org>) and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively. We have revised the methods and results sections of the manuscript as follows to provide the detailed information of screening procedures and supplement the gene lists (Supplementary Table 3). Additionally, we revised the result section as follows to clarify the screening procedures employed in current study.

Methods (Page 27-28, line 811-838):

Computational Analysis

The present study employed the expression matrix and clinical information of glioma samples from four RNA-seq datasets: CMU RNA-seq cohort (<https://ngdc.cncb.ac.cn/gsa-human/s/3qyKeG8n>), the Chinese Glioma Genome Atlas (CGGA) RNA-seq cohorts, including CGGA325 and CGGA693 cohorts (<http://www.cgga.org.cn>), and the Cancer Genome Atlas RNA-seq cohort (TCGA cohort, <https://tcga-data.nci.nih.gov/tcga/>) (Supplementary Table 1). The ssGSEA R package was used to

calculate four metabolic mode scores of each patient in indicated datasets based on corresponding metabolic gene-sets (Glycolysis, oxidative phosphorylation, fatty acid, and glutamine metabolism) downloaded from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) (Supplementary Table 2).

GSEA was performed to explore whether the identified sets of genes showed statistical differences between indicated groups. Gene sets were submitted to the DAVID website (<https://david.ncifcrf.gov/>) to perform gene ontology and pathway annotation. GSE131928 GBM, GSE139448 GBM, and GSE171891 glioma datasets were applied for detecting the expression of HEXB in GBM and non-malignant cell populations. There are 8 GBM samples included in GSE117891 glioma dataset, which were subjected for the analysis. GSE5099 dataset was used for detecting the expression of HEXB in different status of macrophages or monocytes. The t-distributed stochastic neighbor embedding (TSNE) analysis with R programming language was employed to access expression patterns of grouped cells in GBM. Limma R package was performed to identified the DEGs between two different groups ($\text{LogFC} > 0.5$ and $P \text{ value} < 0.05$). Macrophage score reflecting relative macrophage proportions was calculated based on the TIMER2.0 (<https://timer.cistrome.org/>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively. Pearson correlation analysis was used to compare the correlation between glycolysis score and macrophage ratio calculated by two algorithms.

Results (Page 6-8, line 111-182):

HEXB is an unfavorable indicator which facilitates the regulation of glioma cell glycolysis associated with TAMs

To characterize metabolic promoting signaling in grade IV glioma with divergent IDH1 status, we enrolled grade IV glioma samples from four glioma RNA-seq cohorts (CMU cohort $n = 30$, CGGA325 cohort $n = 139$, CGGA693 cohort $n = 239$, TCGA cohort $n = 148$; Supplementary Table 1) including 556 cases of grade IV gliomas (IDH1 wild type = 453; IDH1 mutant = 103) for analysis. The single-sample gene set enrichment analysis (ssGSEA) algorithms based on representative gene sets of four major metabolic modes, including glycolysis, oxidative phosphorylation, fatty acid and glutamine metabolism (Supplementary Table 2), were used for evaluating the signaling activation of these metabolic modes in grade IV glioma. Then, glycolysis was identified as the metabolic mode with most diverse activation status between IDH1 wild type glioma (Glioblastoma, GBM) and grade IV IDH1 mutant glioma compared to other three metabolic modes (Fig. 1a). We further validated the existed difference in glycolysis levels between grade IV IDH1 mutant and GBM in both male and female patient groups in CGGA325 and CGGA693 cohorts, which indicating the difference in glycolysis levels caused by IDH1 mutation status independent of sex (New Supplementary Fig. 1a, b; CMU and TCGA cohorts only include one and two female patients with IDH1 mutation, respectively. Thus, the metabolic capacity of different IDH1 mutation status cannot be statistically determined based on gender). Additionally, we evaluated the sex ratio of glioma patients in the above four glioma datasets and found that the ratio of males to females did not exceed 2:1 (Supplementary Fig. 1e). Meanwhile, we found that sex was not a key factor leading to poor prognosis in glioma patients through univariate Cox (Supplementary Fig. 1f-h) and Kaplan Meier analysis (Supplementary Fig. 1i-k) (CGGA325, CGGA693 and TCGA cohorts; Since the survival information of CMU RNA-seq dataset in Fig. 1a isn't available currently, this analysis didn't include CMU RNA-seq dataset.). Subsequently, we compared the correlation between glycolysis

activation and the infiltration of macrophages represented by the above calculated glycolysis score and macrophage scores based on Timer¹² and Xcell algorithms, respectively. We found that the upregulation of glycolysis score was significantly positively correlated with the elevation of macrophage scores. This indicates the increased infiltration of macrophages accompanying with enhanced glycolysis in GBM (Fig. 1b) and suggests the important role of tumor associated macrophages (TAMs) in promoting the glycolytic activity of GBM. Then, we investigated the function and regulatory process of glycolysis in the GBM microenvironment and compared the glycolysis activation status of malignant cells and TAMs in three single-cell RNA-sequencing GBM dataset (scRNA-seq datasets: GSE117891, GSE131928, and GSE139448) with the glycolysis score. We found that GBM cells had a significantly higher glycolysis score than TAMs (Supplementary Fig. 2a-c), which indicated that glycolysis may be more activated in malignant cells than TAMs in GBM.

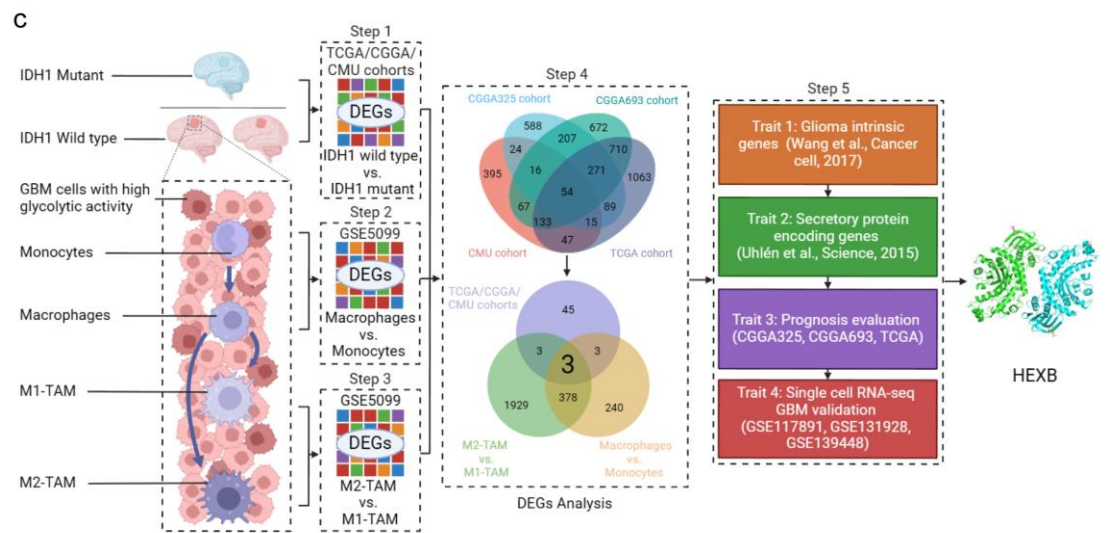
Next, we sought to screen the crucial regulator in GBM facilitating cancer cell glycolysis associated with TAMs, which is not only expressed by both of GBM cells and TAMs, but also could potentially exhibit its function as secretory protein mediating the crosstalk between GBM cells and TAMs (Fig. 1c). Firstly, we examined differentially expressed genes (DEGs) between GBM and IDH1 mutant grade IV glioma in the above four glioma cohorts, respectively (CMU, CGGA325, CGGA693 and TCGA; Step 1). A total of 54 gene candidates (DEG1, Supplementary Table 3) were obtained from the intersection of these 4 datasets, which were significantly overexpressed in GBM. Secondly, we intersected DEG1 with a characteristic gene list of TAMs summarized from monocyte to macrophage transformation (Step 2) and M2 versus M1 macrophages (Step 3), both of which based on DEG analysis (DEG2, Supplementary Table 3) of a macrophage maturation and polarization dataset (GSE5099). The intersection of DEG1 (n = 54) and DEG2 (n = 381) resulted in 3 genes (SIPA1L1, GUSB and HEXB, Supplementary Table 3) (Step 4). Thirdly, given that the critical role of cancer cell-macrophage crosstalk in driving GBM cells glycolysis addiction and macrophages transformation, we sought to examine the regulator with elevation in both of GBM cells and TAMs and potentially involved in driving glycolysis addiction and tumorigenesis through inter- and intra-cellular mechanisms^{13, 14}. We intersected these 3 genes with a glioma intrinsic gene list (Step 5 Trait 1)¹⁵ and a secretory protein related gene list (Step 5 Trait 2)¹⁶ (Supplementary Table 3). There were two candidates (GUSB and HEXB) meeting this criterion. Then, through prognostic analysis in CGGA and TCGA, we found that HEXB showed a stronger correlation with poor prognosis of GBM and glioma patients compared to CUSB (Step 5 Trait 3, Supplementary Table 4 and Supplementary Fig. 3), and its elevation served as a risk factor for GBM survival independent of sex (Supplementary Fig. 4). Finally, we examined three single-cell RNA-seq GBM datasets, which demonstrated HEXB highly-expressed in both of malignant cells and TAMs in GBM (GSE117891, GSE131928, and GSE139448; Step 5 Trait 4), while its expression was significantly upregulated in GBM compared to grade IV IDH1 mutant glioma (Supplementary Fig. 5). Therefore, we chose HEXB as the research target, which may serve as a potential determinant for TAMs-enhanced malignant cell glycolysis in GBM (Fig. 1c).

References

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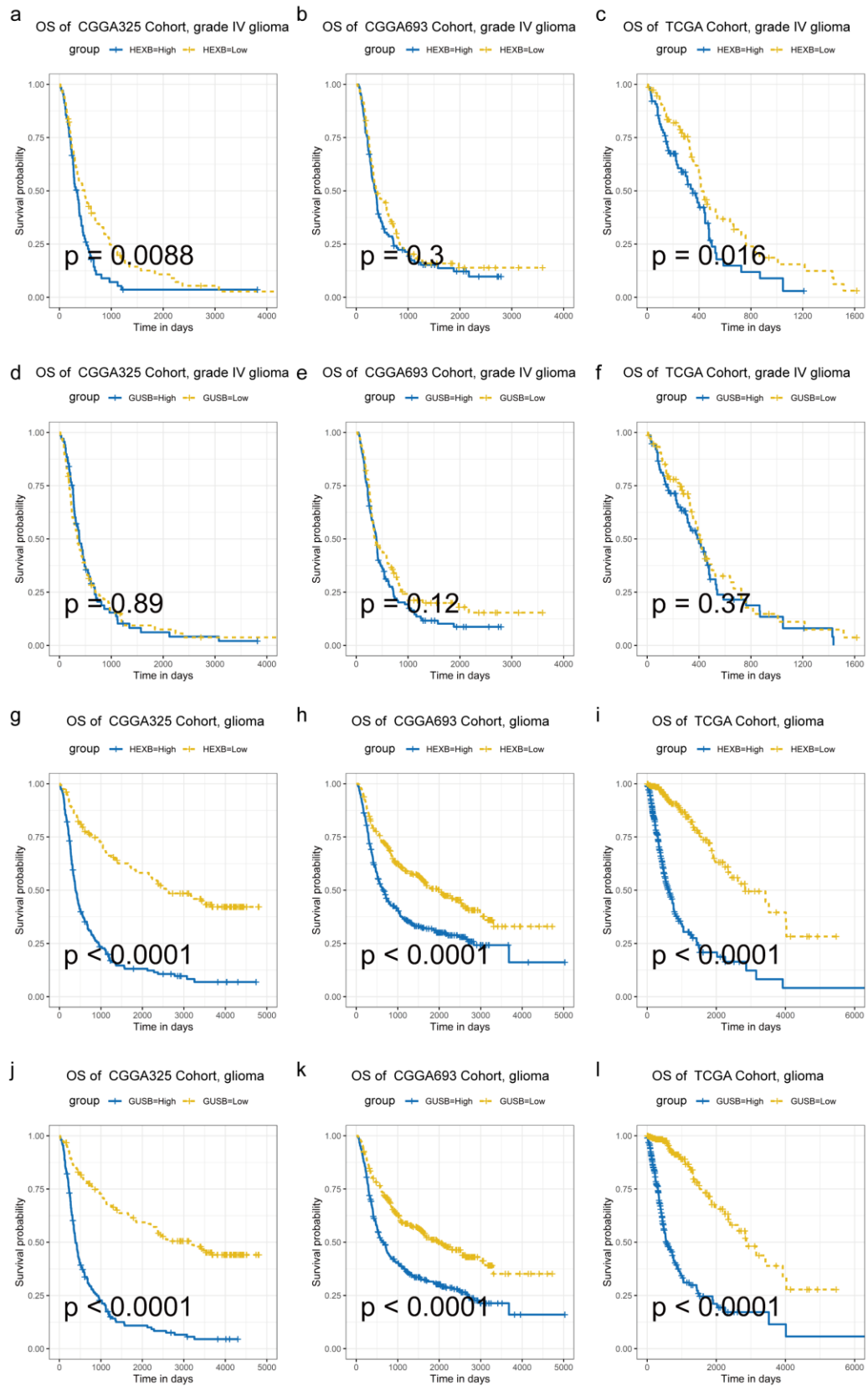
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Revised Fig. 1c



Revised Fig. 1c: The filtering flow diagram showing the profiling of potential microenvironment related glycolytic targets in indicated GBM cohorts, which identified HEXB as the potential secretory target and malignant and macrophage co-expressed gene in IDH1 wild type glioma.

New Supplementary Fig. 3



Supplementary Fig. 3. The survival analysis in indicated glioma and grade IV glioma according to HEXB and GUSB expression in indicated glioma datasets.

a-f Overall survival curves stratified by HEXB (a-c) and GUSB (d-f) expression in indicated three grade IV glioma cohorts, respectively (Log-rank analysis; a, d: CGGA 325 n = 137, including low group n = 68 and high group n = 69; b, e: CGGA 693 n = 237, including low group n = 118 and high group n = 119; c, f: TCGA n = 152, including low group n = 76 and high group n = 76).

g-l Overall survival curves stratified by HEXB (g-i) and GUSB (j-l) expression in indicated three glioma cohorts (Log-rank; g, j: CGGA 325 n = 313, including low group n = 156 and high group n = 157; h, k: CGGA 693 n = 657, including low group n = 328 and high group n = 329; i, l: TCGA cohort n = 603, including low group n = 301 and high group n = 302).

*1.1 If it is bulk RNAseq, the correlation between “glycolysis score” and “macrophage score” (again, the definition of these are not clear) can be explained by that in some samples, macrophage percentage is higher, and macrophage tends to be more glycolytic than other cell types, such as GBM, rather than glycolysis in IDH1 WT GBM is higher, *and* it is associated with more TAMs. This is important because there is some evidence in the literature that high glycolysis, or Warburg effect, observed in solid tumor is largely driven by the immune cells in the tumor rather than cancer cells. (e.g. Reinfeld et al, Nature 2021)*

Response: We appreciate this precious comment and apologized for the insufficient description of “glycolysis score” and “macrophage score” definition. The glycolysis score and the scores of the other three metabolic modes (oxidative phosphorylation, fatty acid, and glutamine metabolism) were established with ssGSEA method and corresponding gene-set obtained from Gene Set Enrichment Analysis website (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>; listed in Supplementary Table 2). Macrophage score reflecting relative macrophage proportions were calculated based on the TIMER2.0 (<https://timer.cistrome.org>)¹² and Xcell (<https://aran-lab.com/software/xcell/>) algorithm, respectively.

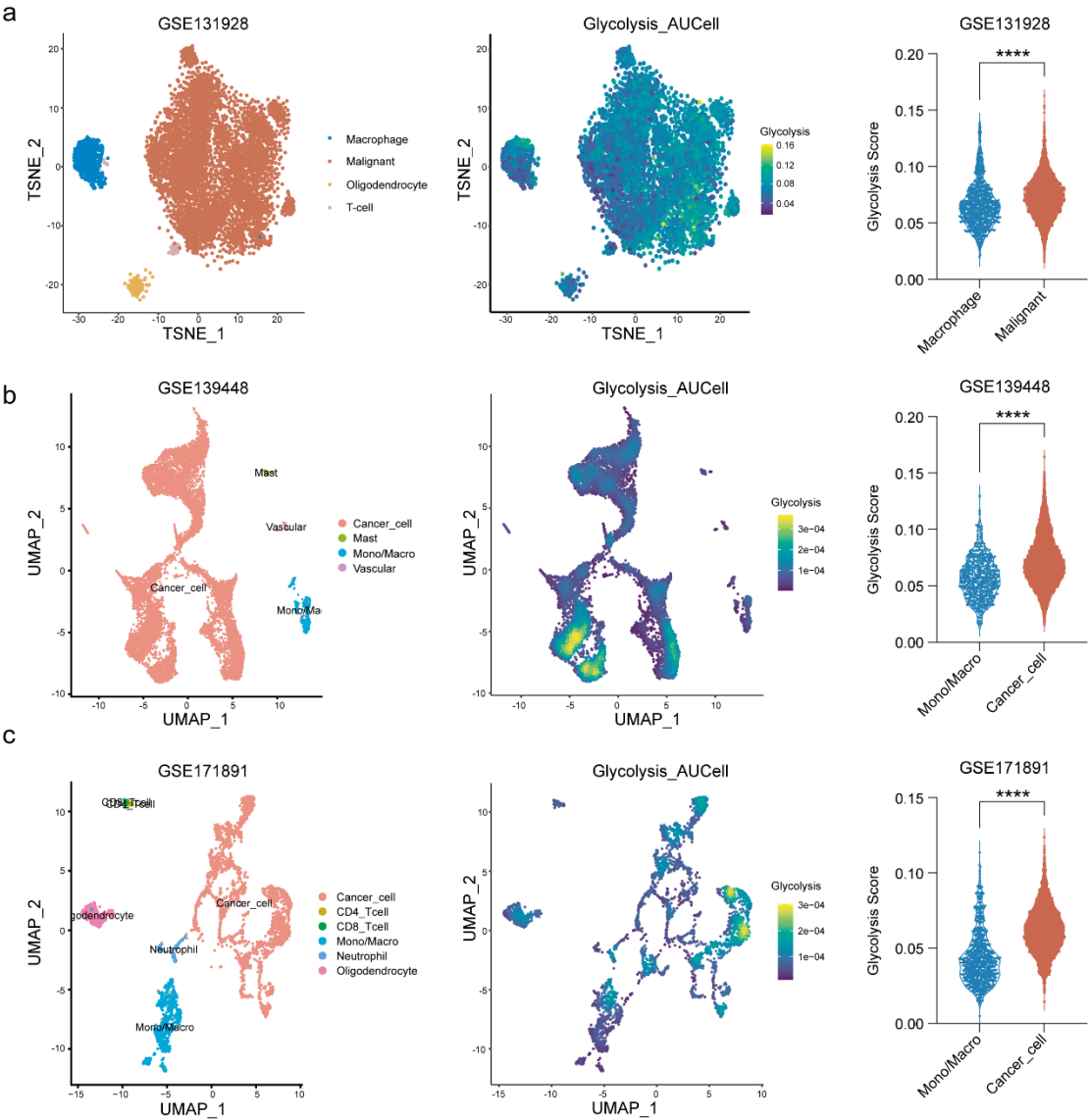
According to Reinfeld’s paper (*Nature*. 2021. **PMID: 33828302**), different cell components consume certain nutrients according to the program and distribute nutrients between cells. Cancer cells absorb glutamine and fatty acids, while immune cells absorb glucose. This study employed the ccRCC, colonic adenocarcinoma and breast cancer model to illustrate their conclusion, and the metabolic patterns of tumors and immune cells in GBM were not included. In GBM TME, glycolysis is a major metabolism mode involved in the behavior regulation of malignant cells like radioresistant GBM cells and macrophage transformation (*J Exp Clin Cancer Res*. 2021. **PMID: 34488821**; *Immunity*. 2024. **PMID: 38703775**). In this study, we focus on exploring the differences in metabolic modes between IDH1 wild type (GBM) and grade IV IDH1 mutant glioma, presenting that glycolysis was the most significant differential metabolic mode between GBM and grade IV IDH1 mutant patient samples (Fig. 1a). According to this comment, we further investigated the function and regulatory of glycolysis process in the GBM microenvironment and compared the glycolysis activation status of malignant cells and TAMs in three single-cell RNA-sequencing GBM dataset with the glycolysis score employed in Fig. 1a analysis, which is established by AUCell method with a glycolysis gene-set downloaded from GSEA website (<http://www.broadinstitute.org/gsea/index.jsp>, Supplementary Table 1). We found that malignant cells had a significantly higher glycolysis score than TAMs in GBM (New Supplementary Fig. 2a-c), which indicated that glycolysis may be more activated in GBM cells than TAMs. We have added these data (New

Supplementary Fig. 2a-c) to the revised manuscript and revised the text in result section accordingly (Page 7, line 148-155).

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12. Li T, et al. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic acids research* 48, W509-w514 (2020).
PMID: 33828302. Reinfeld BI, et al. Cell-programmed nutrient partitioning in the tumour microenvironment. *Nature* 593, 282-288 (2021).
PMID: 34488821. Kang H, et al. Downregulated CLIP3 induces radioresistance by enhancing stemness and glycolytic flux in glioblastoma. *Journal of experimental & clinical cancer research: CR* 40, 282 (2021).
PMID: 38703775. De Leo A, et al. Glucose-driven histone lactylation promotes the immunosuppressive activity of monocyte-derived macrophages in glioblastoma. *Immunity* 57, 1105-1123.e1108 (2024).

New Supplementary Fig. 2



New Supplementary Fig. 2, related to Fig. 1 The comparative analysis based on glycolytic scores of macrophages and malignant cells in single-cell GBM RNA-sequencing datasets.

a-c Different cell annotations (Left), glycolysis score calculation and distribution in indicated cell components based on AUCell method (Middle), and the comparative analysis based on glycolysis scores of GBM cells and mono/macrophages (Right, t-test) in GSE131928 (a), GSE139448 (b) and GSE171891 (c) scRNA-seq GBM cohorts (GSE131928 n = 7930: Malignant (GBM cells) n = 6863; Macrophage n = 754, SMART-seq2; GSE139448 n = 12444: Cancer cell (GBM cells) n = 11714, Monocyte/Macrophage n = 535, 10x Genomics; GSE171891 n = 4193, 8 GBM sample data were included for the analysis: Cancer cell (GBM cells) n = 2611, Monocyte/Macrophage n = 742, 10x Genomics).

(**** $P < 0.0001$).

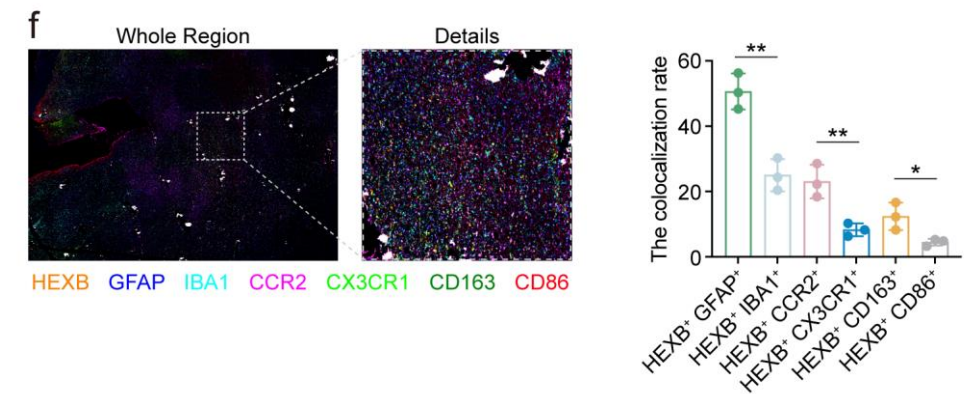
2. It is unclear how is “glioma intrinsic gene” defined. If it is from ssRNA seq, the exact method needs to be better described. From the results later, it seems that not only glioma cells express HEXB. It would be helpful to assess what percentage of HEXB in tumor microenvironment is GBM cells -originated. To answer this question, it would also be helpful to understand what portion of the tumor is GBM cells, and TAMs. Flow or ssRNA-seq can give this result.

Response: We thank the reviewer for this valuable comment. We apologize for the inadequate description of the definition of “glioma intrinsic gene”, which was defined according to a previous report¹⁵. Glioma intrinsic genes refers to the gene with intrinsic expression in glioma cells, while they may also be expressed by non-malignant cells in the TME. Indeed, as a glioma intrinsic gene, HEXB has a certain abundance of expression in glioma associated macrophages. This was supported by our data obtained from tissue polychromatic fluorescence staining (Revised Fig. 1f) and the analysis of HEXB expression patterns in three GBM single cell RNA-seq datasets (New Supplementary Fig. 5i-k). Therefore, according to this comment, we have made the following revision to the manuscript and added the reference (Page 7, line 170) and the above revised and new figures (Revised Fig. 1f and new Supplementary Fig. 5i-k) to define the intrinsic genes of glioma and the expression pattern of HEXB in GBM accordingly.

Reference

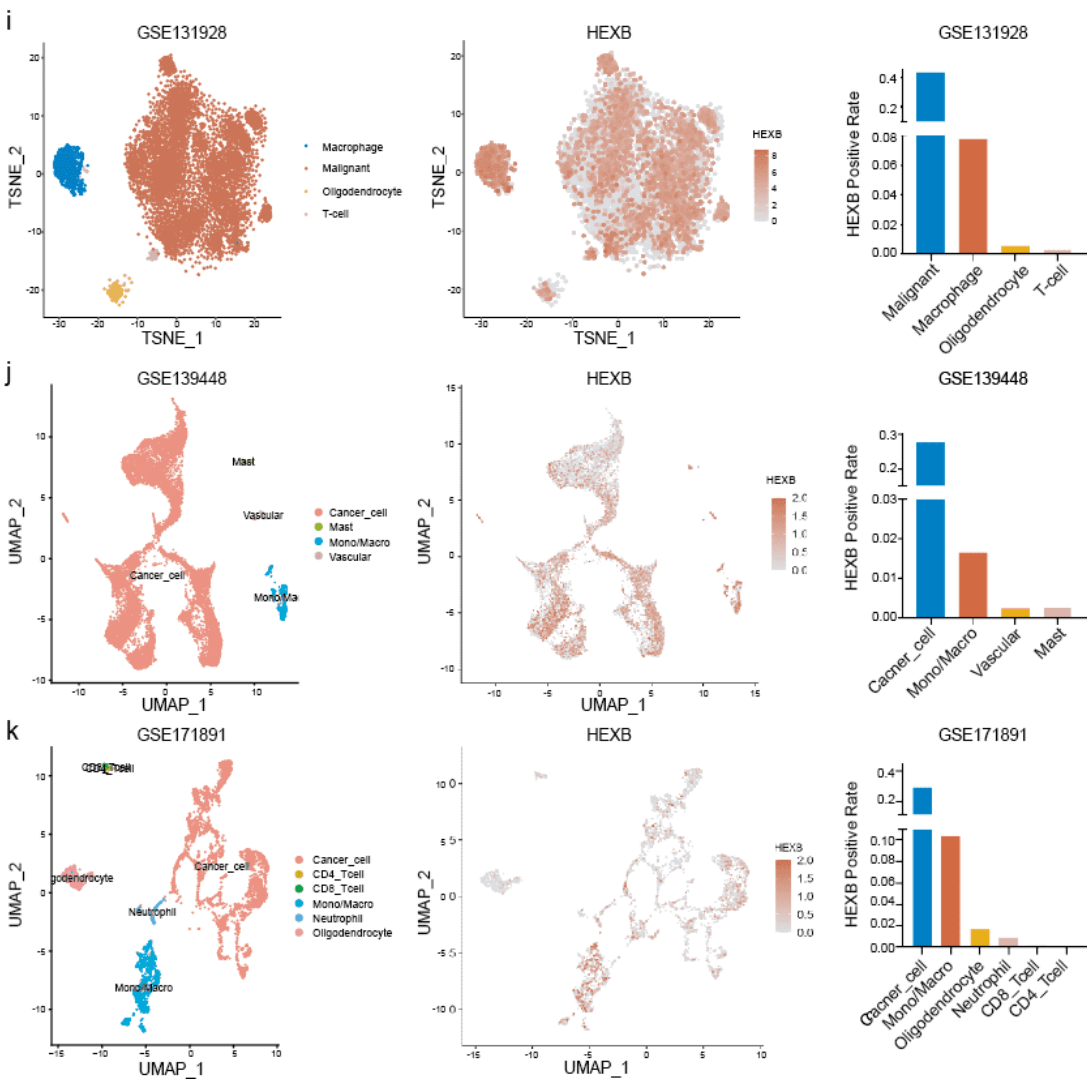
15. Wang Q, et al. Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. Cancer cell 32, 42-56.e46 (2017).

Revised Fig. 1f



Revised Fig. 1f: Representative multi-color Immunofluorescence co-staining images (Left and middle) and analysis (Right) of HEXB, IBA1, CCR2, CD86, CD163, and GFAP in clinical GBM tissue samples.

New Supplementary Fig. 5i-k, related to Fig. 1



New Supplementary Fig. 5

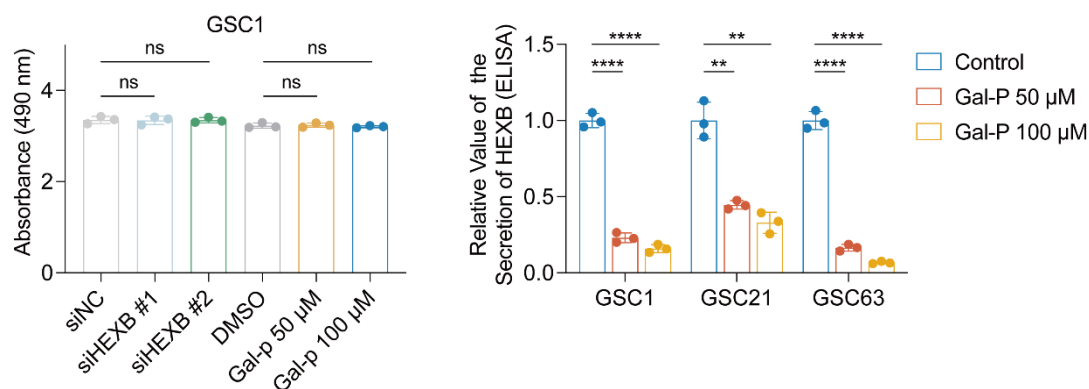
i-k Different cell annotations (Left), and the proportion of HEXB positive cells (Count number > 0) (Middle) and the comparative analysis based on HEXB expression in indicated cell populations (Right, *t*-test) of GBM (i: GSE131928 scRNA-seq GBM cohort (n = 7930): Malignant (GBM cells) n = 6863, Macrophage n = 754, Oligodendrocyte n = 219, T cell n = 94, SMART-seq2; j: GSE139448 scRNA-seq GBM cohort (n = 12444): Cancer cell (GBM cells) n = 11714, Monocyte/Macrophage n = 535, Vascular n = 100, Mast n = 95, 10x Genomics; k: GSE171891 scRNA-seq glioma cohort (n = 4193, 8 GBM sample data were included for the analysis): Cancer cell (GBM cells) n = 2611, Monocyte/Macrophage n = 742, Oligodendrocyte n = 342, Neutrophil n=134, CD8 T cell n=45, CD4 T cell n = 41, unknown n = 278, 10x Genomics).

3. Detail is also needed for seahorse experiments. How long was the cells in seahorse plate? Given that HEXB is a secretory protein, if the cells are just seeded with fresh media, HEXB is unlikely to accumulate to meaningful amount in the extracellular space. So what is the mechanism? If the cells are seeded for some time to allow HEXB accumulation (a measurement of HEXB level in media would be helpful), then how is the impact of different proliferation rates adjusted? Are the seahorse results normalized to live cell number in each condition?

Response: We appreciate this valuable comment. Seahorse experiments were performed after 24h hours of the cells lay into the plate. As shown in Previous Supplementary Fig. 2G and H, MTS assays demonstrates that the promotion of HEXB on the proliferation of GBM cells is basically observed after 48 hours of seeding the cell to the plate. Thus, it could be negligible for the proliferation differences among different groups when detecting glycolysis on the machine at 24 hours after laying the sample into the board. In addition, we lay boards with 20000 cells per well and we observed that there was no significant difference in cell count results among the groups through MTS analysis after final testing (Reviewer only Fig. 4 Left panel and Reviewer only Table 1).

In order to examine the difference of HEXB secretion in each sample group, we employed an ELISA kit to detect the secretion content of HEXB in the cell supernatant after cell lay into the plate. We found that the endogenous intervention HEXB group was compared with the control group, there is a statistically significant difference in the secretion of HEXB in the cell supernatant (Reviewer only Fig. 4 Right panel).

Reviewer only Fig. 4



Reviewer only Fig. 4:

Left MTS analysis of indicated representative GBM cells after seahorse testing (GSC1; n = 3, t-test).

Right ELISA analysis of HEXB protein secretion in indicated GBM cells treated with DMSO or Gal-P (GSC1, GSC21 and GSC63; n = 3, t-test).

Reviewer only Table 1: We applied the MTS assay of indicated GSC groups after seahorse testing, and the absorbance of 490 nm can indirectly reflect the relative number of cells in each group, there is no significant statistical difference in absorbance between any two groups in the same batch of experiments.

MTS Absorbance (490 nm) of indicated groups						
Cell line	Group	N1	N2	N3	N4	N5
GSC1	siNC	3.362	3.428	3.268	3.239	3.315
GSC1	siHEXB #1	3.243	3.416	3.374	3.482	3.336
GSC1	siHEXB #2	3.302	3.328	3.412	3.263	3.307
GSC1	DMSO	3.293	3.188	3.203	3.311	3.287
GSC1	Gal-p 50 μ M	3.284	3.237	3.194	3.158	3.226
GSC1	Gal-p 100 μ M	3.182	3.205	3.228	3.303	3.326
GSC21	siNC	3.151	3.047	3.203	/	/
GSC21	siHEXB #1	3.018	3.113	3.193	/	/
GSC21	siHEXB #2	3.182	3.208	3.124	/	/
GSC21	DMSO	3.095	2.996	3.104	/	/
GSC21	Gal-P 50 μ M	2.983	3.037	3.102	/	/
GSC21	Gal-P 100 μ M	3.117	3.079	3.173	/	/
GSC63	siNC	3.634	3.612	3.726	/	/
GSC63	sgHEXB #1	3.537	3.562	3.601	/	/
GSC63	sgHEXB #2	3.628	3.573	3.682	/	/
GSC63	DMSO	3.437	3.402	3.396	3.128	/
GSC63	Gal-P 50 μ M	3.417	3.338	3.394	3.225	/
GSC63	Gal-P 100 μ M	3.307	3.328	3.368	3.178	/
GSC1	Vec+DMSO	3.225	3.233	3.264	/	/
GSC1	Vec+Gal-P 50 μ M	3.257	3.239	3.302	/	/
GSC1	Vec+Gal-P 100 μ M	3.311	3.285	3.227	/	/
GSC1	5SA+DMSO	3.346	3.338	3.426	/	/
GSC1	5SA+Gal-P 50 μ M	3.274	3.268	3.317	/	/
GSC1	5SA+Gal-P 100 μ M	3.309	3.284	3.317	/	/
GSC21	Vec+DMSO	3.127	3.048	3.029	3.218	3.183
GSC21	Vec+Gal-P 50 μ M	3.023	2.996	2.873	2.864	3.103
GSC21	Vec+Gal-P 100 μ M	2.883	2.918	3.003	3.126	3.017
GSC21	5SA+DMSO	3.136	3.118	3.072	2.938	2.892
GSC21	5SA+Gal-P 50 μ M	3.049	3.126	2.969	3.038	3.152
GSC21	5SA+Gal-P 100 μ M	3.113	3.027	2.986	3.231	3.194
GSC1	Control	3.427	3.376	3.285	/	/
GSC1	Verteporfin	3.269	3.332	3.228	/	/
GSC1	Verteporfin+rhHEXB 0.5 μ g/ml	3.341	3.327	3.203	/	/
GSC1	Verteporfin+rhHEXB 1 μ g/ml	3.286	3.106	3.322	/	/

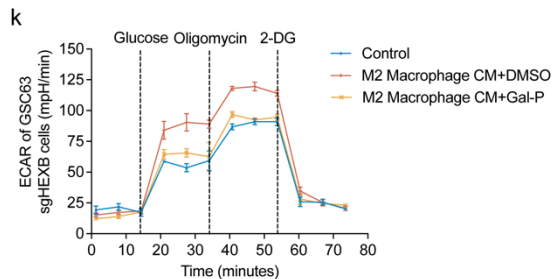
GSC21	Control	2.936	3.012	3.117	3.263	3183
GSC21	Verteporfin	3.205	3.078	2.995	3.152	3.218
GSC21	Verteporfin+rhHEXB 0.5 μ g/ml	2.984	3.012	3.224	2.936	3.215
GSC21	Verteporfin+rhHEXB 1 μ g/ml	3.125	3.106	2.992	3.227	3.158
GSC63 sgHEXB	GSC63 control	3.638	3.727	3.883	/	/
GSC63 sgHEXB	GSC63 rhHEXB	3.693	3.753	3.609	/	/
GSC63 sgHEXB	GSC63 rhHEXB+Anti-ITGB1	3.538	3.647	3.771	/	/
GSC1	IDH1 WT+DMSO	3.116	3.327	3.083	/	/
GSC1	IDH1 WT+Gal-P	3.039	3.127	3.338	/	/
GSC1	IDH1 RG132H+DMSO	3.094	3.173	3.206	/	/
GSC1	IDH1 RG132H+Gal-P	3.083	3.182	3.336	/	/
GSC1 shHEXB	GSC1 control	3.227	3.029	3.182	3.324	3.183
GSC1 shHEXB	GSC1 rhHEXB 0.5 μ g/ml	3.093	3.274	3.028	2.976	3.112
GSC1 shHEXB	GSC1 rhHEXB 1 μ g/ml	3.128	3.331	3.269	3.108	3.238
GSC21 shHEXB	GSC21 control	2.978	3.117	3.028	2.883	2.985
GSC21 shHEXB	GSC21 rhHEXB 0.5 μ g/ml	2.883	2.957	3.083	3.069	3.106
GSC21 shHEXB	GSC21 rhHEXB 1 μ g/ml	3.119	2.927	2.804	3.038	3.126
GSC1 shHEXB	siNC+BSA	2.946	2.983	3.016	/	/
GSC1 shHEXB	siNC+rhHEXB	2.992	3.083	3.091	/	/
GSC1 shHEXB	siITGB1+BSA	3.117	3.102	3.203	/	/
GSC1 shHEXB	siITGB1+rhHEXB	3.094	2.993	3.121	/	/
GSC1 shHEXB	siILK+BSA	3.096	3.128	3.203	/	/
GSC1 shHEXB	siILK+rhHEXB	3.112	3.202	3.227	/	/
GSC1	siNC+rhHEXB	3.124	3.093	3.152	2.983	2.991
GSC1	siHIF1 α +BSA	3.096	2.917	3.118	3.102	3.203
GSC1	siHIF1 α +rhHEXB 0.5 μ g/ml	3.227	3.103	3.028	3.119	3.181
GSC1	siHIF1 α +rhHEXB 1 μ g/ml	3.136	3.173	3.204	3.096	3.125
GSC63	Control	3.036	3.009	2.886	/	/
GSC63	M0 macrophage CM	2.896	2.931	3.016	/	/
GSC63	M1 macrophage CM	3.149	3.131	3.085	/	/
GSC63	M2 macrophage CM	3.062	3.139	2.982	/	/
GSC63 sgHEXB	Control	3.126	3.04	2.999	/	/
GSC63 sgHEXB	M2 macrophage CM+ DMSO	3.404	3.389	3.298	/	/
GSC63 sgHEXB	M2 macrophage CM+ Gal-P	3.282	3.345	3.241	/	/
SB	IDH1 WT+DMSO	3.354	3.343	3.216	/	/
SB	IDH1 WT+Gal-P	3.299	3.119	3.335	/	/

SB	IDH1 RG132H+DMSO	2.949	3.025	3.138	/	/
SB	IDH1 RG132H+Gal-P	3.218	3.091	3.008	/	/
GSC24	DMSO	2.953	2.974	3.092	3.113	3.137
GSC24	Gal-P 50 μ M	2.864	2.938	3.044	3.182	2.993
GSC24	Gal-P 100 μ M	2.903	3.083	3.129	3.047	3.168

4. The claim that “TAMs recruited by cancer-HEXB feedback enhances GBM cells glycolysis” is not well supported. Specific evidence is needed to show that recruited TAMs directly promote glycolysis in GBM cells. The mechanism by which TAMs could enhance GBM cells glycolysis is also unclear.

Response: We thank the reviewer for this valuable comment. According to this comment, we examined whether M2 macrophages could enhance the glycolysis rate of GBM cells through co-culture experiments (GBM cells cocultured with CM obtained from M2 macrophages). The results demonstrates that M2 macrophages can express and secrete more HEXB than M0 macrophages (Previous Fig. 6h-j and Supplementary Fig. 7e), which could directly promote the glycolysis of GBM cells (Previous Supplementary Fig. 7a-d). To further support this conclusion, we added HEXB inhibitor Gal-P into the co-culture system of GSC63 sgHEXB cells and M2 macrophages. The result demonstrated that M2 macrophages significantly increased the glycolysis rate of GSC63 cells transduced with sgHEXB vector, which could be partially abolished by the supplement of HEXB inhibitor Gal-P (New Supplementary Fig. 15k). Therefore, it is reasonable to infer that the mechanism of TAM enhancing glycolysis of GSCs is partly due to the secretory HEXB supplied by M2 macrophages.

New Supplementary Fig. 15k, related to Fig. 6



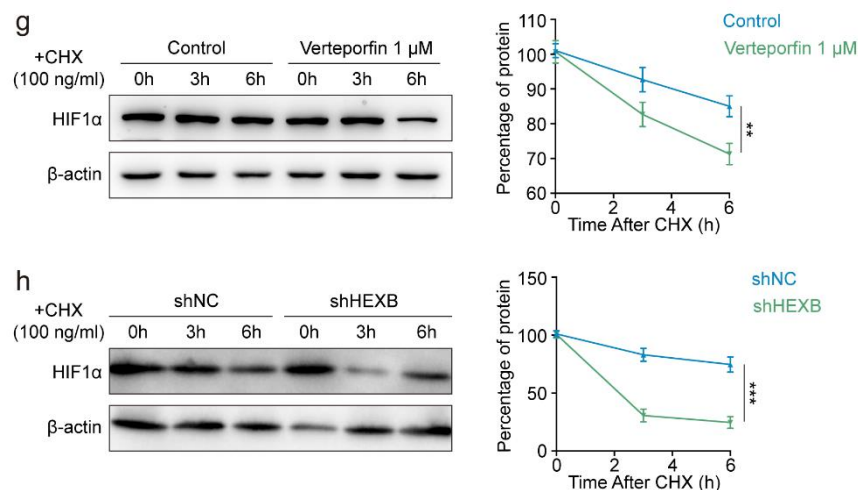
New Supplementary Fig. 15k: The analysis of glycolytic rate (ECAR) of sgHEXB GBM cells (GSC63) with treatment of conditioned medium (CM) obtained from THP-1 derived M2 macrophages or HEXB inhibitor Gal-P (100 μ M) (n = 3, t-test).

5. In the HIF section, the authors claimed that “HIF stability is reduced with YAP1 inhibition”. However, it is unclear how Figure 5 measures HIF “stability” rather than level.

Response: We apologize for the trouble caused by the imperfect presentation of our data. In the original version of the manuscript, we only showed the expression of HIF1 α at indicated time points (Previous Fig. 5G and H), but did not show the degradation curve, resulting in this misunderstanding. In this revision, we quantitatively analyzed the expression of HIF1 α at different time points in each

group after verteporfin (YAP1 inhibitor) treatment through plotting the degradation curve, and measured the protein stability through comparing the degradation rate (New Fig. 5g and h).

New Fig. 5g and h



New Fig. 5

g Western blotting analysis of HIF1 α protein stability in GSC1 samples with indicated verteporfin or DMSO treatment (n = 3, t-test).

h Western blotting analysis of HIF1 α protein stability in GSC1 transduced with shNC or shHEXB vector, respectively (n = 3, t-test).

6. To validate the pro-glycolytic effect of HEXB is through HIF, it would be clearer to directly report the transcript levels of specific HIF target genes in glycolysis, and how they are affected when HIF is silenced, and whether their expression in shHEXB can be rescued when cells are treated with HIF stabilizer. It is not very clear how “index of HIF transcriptional activity” was defined or measured.

Response: Thank you very much for this valuable comments. Based on this suggestion, we improved the relevant experiments with the detection of PKM2 and HK2 by RT-qPCR. These two enzymes are not only well-known downstream targets of HIF1 α , but also functions as key enzymes of glycolysis process^{33, 34, 35, 36, 37}. The data showed that the expression of PKM2 and HK2 at transcription levels were significantly decreased with genetic disruption of HEXB in GBM cells, and treatment with the HIF1 α activator CoCL2 reversed the downregulation of PKM2 and HK2 induced by HEXB knockdown (New Supplementary Fig. 14p). Furthermore, for detecting the HIF transcriptional activity, a dedicated HIF1 α transcriptional activity assay kit was used for the measurement (Cayman, 10006910), which demonstrated that the transcriptional activity of HIF1 α in IDH1 wild type glioma cells were significantly higher than that in IDH1 mutant glioma cells or glioma cells transfected with IDH1 mutant vector (New Supplementary Fig. 14o). We have revised the Methods and Results as follows:

Methods (Page 27, line 802-809)

Detection of the HIF1 α transcriptional activity

The HIF1 α transcriptional activities were detected by a dedicated HIF1 α transcriptional activity assay kit (Cayman, 10006910), following the manufacturer's instructions. Briefly, after nuclear protein extraction and quantification, 50 μ g nuclear protein samples were added to each well and followed by supplementing a mixed and uniform reagent kit working solution. Then, the absorbance values were measured with an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm, which reflects the relatively high or low transcription activities of HIF1 α in indicated groups.

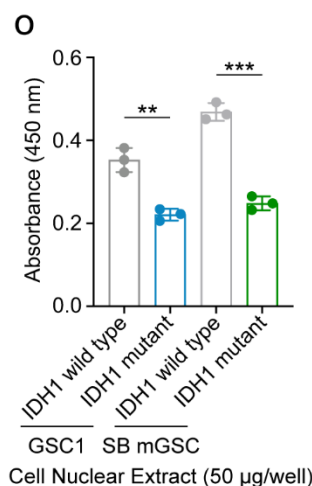
Results (Page 15, line 417-426)

Secondly, for detecting the HIF transcriptional activity, a dedicated HIF1 α transcriptional activity assay kit (Cayman, 10006910) was used for measuring the HIF transcriptional activity, which demonstrated that the transcriptional activity of HIF1 α in IDH1 wild type GSC1 and mGSCs were significantly higher than that in GSC1 transfected with IDH1 mutant vector and IDH1 mutant mGSCs, respectively (Supplementary Fig. 14o). Thirdly, disrupting HEXB in IDH wild type GBM cells efficiently restrained the expression of PKM2 and HK2, two HIF1 α downstream targets^{33, 34, 35, 36, 37}, which could be rescued by the supplement of HIF1 α activator, CoCL2 (Supplementary Fig. 14p). These data support a higher activation of HIF1 α in IDH1 wild type GBM cells than IDH1 mutant cells.

References

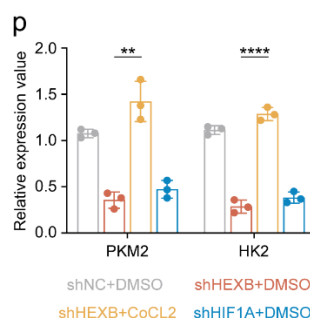
33. Yang W, et al. EGFR-induced and PKC ϵ monoubiquitylation-dependent NF- κ B activation upregulates PKM2 expression and promotes tumorigenesis. *Molecular cell* 48, 771-784 (2012).
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36. Lan F, Qin Q, Yu H, Yue X. Effect of glycolysis inhibition by miR-448 on glioma radiosensitivity. *Journal of neurosurgery* 132, 1456-1464 (2019).
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New Supplementary Fig. 14o, related to Fig. 5



New Supplementary Fig. 14o: The analysis of HIF1 α transcriptional activities in indicated IDH1 wild type and mutant glioma cell samples (n = 3, t-test).

New Supplementary Fig. 14p, related to Fig. 5



New Supplementary Fig. 14p: The qPCR analysis of PKM2 and HK2 mRNA in GSC1 pretreated with DMSO or CoCL₂ (100 µM) and then separately transduced with non-target, shHEXB or shHIF1A vectors (n = 3, t-test).

7. In macrophage migration assay, the potential difference in macrophage survival and proliferation during the 22h period needs to be controlled for.

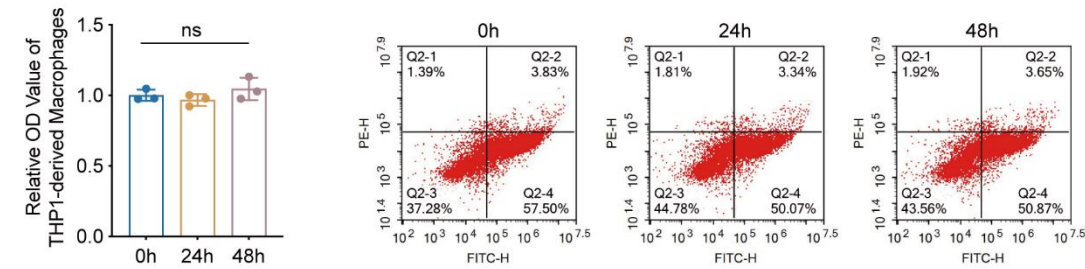
Response: Thank you for this thoughtful suggestion. To address this comment, we conducted MTS and apoptosis tests on THP1-derived macrophages and found no significant differences in the proliferation activity (Reviewer only Fig. 5 left panel) and apoptosis ratio (Reviewer only Fig. 5 right panel) of macrophages within 24-48 hours after induction. This is supported by previous reports, which demonstrates that there is nearly no proliferation observed or even reduced in THP-1 monocytes with PMA treatment (*PLoS One*. 2023. **PMID: 37459313**; *Differentiation*. 2022. **PMID: 36370526**). Therefore, combined with this observation, the impact of differences in macrophage proliferation on migration experiments (Previous Fig. 6C) could be negligible.

References

PMID: 37459313. Liu T, et al. Optimization of differentiation and transcriptomic profile of THP-1 cells into macrophage by PMA. PloS one 18, e0286056 (2023).

PMID: 36370526. Mohd Yasin ZN, Mohd Idrus FN, Hoe CH, Yvonne-Tee GB. Macrophage polarization in THP-1 cell line and primary monocytes: A systematic review. Differentiation; research in biological diversity 128, 67-82 (2022).

Reviewer only Fig. 5



Reviewer only Fig. 5

Left The Relative OD value of THP1-derived macrophages after 0 hours, 24 hours and 48 hours cultured, representing its proliferation ability had no changes (n = 3, t-test).

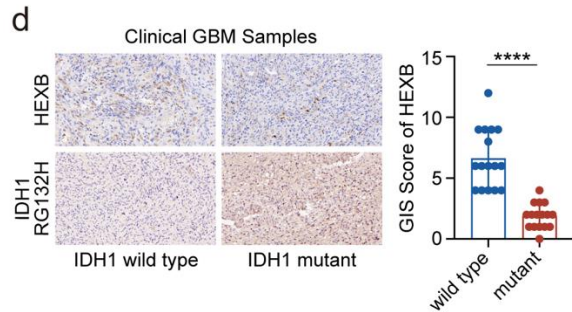
Right The representative images of apoptosis assays in THP1-derived macrophages after 0 hours, 24 hours and 48 hours cultured.

Minor points

1. Figure 1D, the label is unclear what is being probed. For “IDH WT”, the representative image is labeled “IDH1 RG132H”, is that a mislabel?

Response: We appreciate this comment. The labeling of “IDH1 RG132H” in the IHC staining images indicates the representative image of IDH1 RG132H staining in IDH1 wild type and mutant samples (labeled below the images). Indeed, the staining of IDH1 RG132H antibody is almost negative in IDH1 wild type glioma samples (GBM). We have revised the labeling in Fig. 1d accordingly. The revised image was listed below.

Revised Fig. 1d



Revised Fig. 1d: Representative IHC images (Left) and analysis (Right) of HEXB staining in clinical grade IV IDH1 wild type (GBM) and mutant glioma tissue samples (n = 32, including IDH wild type n = 16, IDH mutant n = 16; t-test).

2. There are some grammatical errors and typos throughout. For instance, in the very first sentence of abstract, the word “of” before “cancer” seems to be an error.

Response: We apologize for these errors and have made corrections to the grammar and spelling errors in the corresponding sections, including revising “Glycolytic metabolic reprogramming of cancer” to “Glycolytic metabolic reprogramming in cancer” (Page 3, line 44).

3. It is unclear what the authors mean by “TAMs-associated glioma cell glycolysis”. Some phrasing in this manuscript is confusing.

Response: We thank for this reminder and have made corrections to the unclear phrasing “TAMs-associated glioma cell glycolysis” to “the regulation of GBM cell glycolysis associated with TAMs” in the manuscript (Page 6, line 111-112), to make it clearer for the reviewers and readers.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this revised manuscript, the authors addressed to the comments to the reviewer in detailed manner.

The reviewer has no more concern on this manuscript.

Reviewer #2 (Remarks to the Author):

All of my comments have been addressed. The additional details and methods have greatly improved the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have done an admirable job in addressing reviewer concerns. I have not further comment. The results and conclusions are well supported by the data presented. The significance of the manuscript is that the manuscript investigates both the mechanism of action of HEXB as well as the therapeutic potential of targeting HEXB. This manuscript is anticipated to be of broad interest to the readership of Nature Communications.

Reviewer #4 (Remarks to the Author):

The authors made extensive efforts to address reviewers' comments. My comments have been sufficiently addressed. I recommend acceptance of the paper.

Response to referees

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this revised manuscript, the authors addressed to the comments to the reviewer in detailed manner.

The reviewer has no more concern on this manuscript.

Response: We deeply appreciate your precious comments on our work, which substantially help us improve the quality of present study.

Reviewer #2 (Remarks to the Author):

All of my comments have been addressed. The additional details and methods have greatly improved the manuscript.

Response: We are really grateful for your high evaluation on our manuscript.

Reviewer #3 (Remarks to the Author):

The authors have done an admirable job in addressing reviewer concerns. I have not further comment. The results and conclusions are well supported by the data presented. The significance of the manuscript is that the manuscript investigates both the mechanism of action of HEXB as well as the therapeutic potential of targeting HEXB. This manuscript is anticipated to be of broad interest to the readership of Nature Communications.

Response: Thank you for your valuable comments on our study. We greatly appreciate your recognition of our work and we will make efforts for the clinical translation of HEXB-targeted strategy in GBM.

Reviewer #4 (Remarks to the Author):

The authors made extensive efforts to address reviewers' comments. My comments have been sufficiently addressed. I recommend acceptance of the paper.

Response: Thank you very much for your constructive comments on our study, which greatly assists us strengthen the quality and logic of our work.