

## m6A/IGF2BP3-Driven Serine Biosynthesis Fuels AML Stemness and Metabolic Vulnerability

Corresponding Author: Dr Hengyou Weng

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

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors present a very nice study on how M6A regulatory protein IGF2BP3 is involved in leukemogenesis, which may in part be regulated via control of SPP enzymes PHGDH and PSAT1. Novelty of this research is very high. Yet, some critical points are missed out on or over interpreted, as I will indicate and further explain below.

Comments and advice

The models: It is unclear to me why these AML models were chosen. They are quite uncommon AML cell models. Especially with respect to their metabolic dependency. One representing a FLT3-ITD MOLM13 model, where FLT3-ITD is known to induce glycolysis differently from FLT3 WT cells. U937 cells are lymphoma cells from pleural effusion, which do not represent an AML. Than the MM6 cells with a MLL-AF9 fusion like the mouse model. MLL-rearranged AML are a special type of AMLs. The MLL-rearranged AMLs are very dependent on RAS pathway activation, therefore, often representing RAS mutations a bit more frequently than other AML subtypes.

In general, many genes pop out of the epigenetic screen from which M6A regulatory proteins were cherry picked. What about the other genes? NRAS is part of the top30 genes SG dependent scores, so at least define the difference between NRAS WT and NRAS mutant AML with respect to IGF2BP3 dependency. Maybe comparing these would be more clear with respect to rescue experiments you represent in the paper, where you only see maximum of 50% rescue, appointing that a lot more is going on besides SPP pathway regulation. Serine levels are also only slightly affected by your IGF2BP3 KO guide RNAs. Plus ATF4 is also in your list of IGF2BP3 regulated, so if the SPP pathway regulation partially due to IGF2BP3 regulation and in part indirectly through ATF4 regulation or maybe even only through ATF4 regulation? This should be clarified. Also SIRT4 is in your screen, which is affecting glutamine metabolism and indirectly SPP metabolism. What are NRF2, MYC, ATF4 levels in your models/experiments? Is it all mediated via these transcriptional regulators rather than direct IGF2BP3 effects? In figure 2B, AKG is listed in your TCA but also comes from SPP, form metabolic tracing experiments it should be clear what is coming from where. Furthermore aKG is important for RNA methylation, and might explain your phenotype rather than SPP pathway beyond PSAT1? What I also don't understand is your labeling experiment. If cellular serine is made from glucose, you would obtain M+3 serine. I have never observed these high levels of M+1 serine from labeled 13C-glucose. Where do these come from?

Besides PHGDH and PSAT1 also SHMT2 from the SPP pathway is included as IGF2BP3 hit, why is it excluded? This enzyme converts serine to glycine for further processing toward GSH and nucleotides. To provide a clear overview, all enzymes should be checked and presented if you want to claim these effects are specific for your enzymes of interest. Getting to my next question; What happens downstream of the SPP pathway, what are they using serine for? What is controlled by IGF2BP3 mediated enzyme expression?

I am still puzzled why BP3 KO mice show so little effects. If IGF2BP3 is controlling SPP genes, these mice should present neurologic phenotypes and diseases. So something seems overlooked.

## Reviewer #2

### (Remarks to the Author)

Huang et al present a compelling case that IGF2BP3 is a potential target in AML, especially in combination with SG-deprivation in vitro and in vivo, however, the manuscript often contains biased experiments, often inappropriate controls to prove their hypothesis. Moreover, the title "Augment of Serine Biosynthesis by m6A Modification Drives Leukemogenesis and Stemness as a Metabolic Vulnerability in AML" is inappropriate for this publication given that the study focuses primarily on IGF2BP3, and at points METTL14, but does not consider other fundamental players in m6A biology.

To consider this manuscript for publication, I suggest a change in title, as well as the following:

### Major comments

- 1) In the majority of the in vitro experiments, the authors show only a loss of proliferation, but do not show whether this is caused by cell cycle arrest, differentiation or cell death. This is present throughout the paper and across models (human AML cell lines, murine AML models and patient samples). IGF2BP3 has been reported to cause cell cycle defects in AML, and so this is a significant omission.
- 2) A related point is the L-GMP analyses in Figure 5H/I, where the authors show a loss of L-L-GMPs in the KO mice. Is the loss of IGF2BP3 causing a loss in a leukaemia stem cell marker through apoptosis or cell cycle arrest of these cells, or is it increasing a differentiation marker? There is in fact an increase in Lineage positive markers in the KO mice shown in 5H, can the make-up of the lineage compartment be provided?
- 3) The authors often present selected data from biased approaches to test their hypothesis and reach their conclusions. For example, can the authors explain the reasoning behind using an CRISPR library targeting epigenetic genes only? This library would fail to provide a genome wide analyses of the mechanisms behind SG-deprived AML cells.
- 4) When it comes to the claim in the title that serine biosynthesis is augmented by m6A, the authors only focus on IGF2BP3 and METTL14. Are other key regulators of the m6A modification, e.g. YTHDFs, FTO, ALKBHs also dropped out in the CRISPR-screen? S1F shows the top genes to drop out, where a number are involved in m6A biology, but many are not. Can the authors provide dependency scores of the other key m6A players?
- 5) Although the proportion of m6A modified down-regulated transcripts seems over represented in Figure 2J, since the estimates of the proportion of the genome that is m6A-modified varies between 20-60%, can the authors provide a comparison for this? Perhaps transcripts that are unregulated and unchanged?
- 6) Related in Figure 3H, the m6A methylation activity of METTL14 suggests that KD of METTL14 would result in general decrease in m6A abundance. The m6A -qPCR experiments as a result are very focused, and only concentrate on the transcripts involved in SG biosynthesis, and are therefore biased. It is perhaps likely that a vast number of other transcripts would behave in the same way following METTL14 loss. Can the authors provide comparisons with transcripts to prove this is specific to PSAT1 and PHGDH?
- 7) As with Figure 3H, the authors are conducting very focused experiments, and not looking at the global picture. Can the authors consider looking at other targets, for example with SLAM-seq to properly conclude the mechanism proposed.
- 8) The murine studies in Figure 4 are only conducted using MLL-AF9 driven AML. Is this phenotype seen in other common murine AML models, e.g. NRAS-ETO, FLT3-ITD, PML-RAR $\alpha$ ?
- 9) Can the authors please provide details of the mutational status of the patient samples used in this experiments? Do the samples used contain similar mutations or are they different and are therefore a broad representation of patient biology.
- 10) Regarding the experiments studying normal haematopoiesis in Figure 5, can the authors provide expression data of IGF2BP3 in the stem and progenitor compartments, and differentiated compartments of normal haematopoiesis.
- 11) Considering the anti-AML phenotype upon IGF2BP3 loss are shown most significantly following transplantation, can the authors provide HSC transplantation data for normal haematopoiesis? This will test whether IGF2BP3 KO HSCs are able to give rise to normal blood production following the stress of transplantation.
- 12) Given the impressive data shown with the shRNA experiments + SG-free diet in primary transplant, can the authors perform secondary transplantation with these cells into recipient mice with and without SG-free diet? This will determine if the anti-leukaemic phenotype has memory to the SG-free diet, or would relapsed patients, for example, have to be a SG-free diet long term to be disease free?

### Minor Comments

- 13) Related to the data presented in Figure 4, is there a proliferation defect seen in the murine MLL-AF9 cells, similar to that of the KD cells in THP-1s?

14) It is not clear where the cells for the secondary transplant are coming from. Can the authors include this in a schematic and consider moving these experiments further up the figure with the earlier knockdown experiments?

### Reviewer #3

#### (Remarks to the Author)

The authors identify through CRISPR/Cas9 screen that IGF2BP3 is an important player in the persistence of AML and contributes to satisfying AML's high metabolic demand. By examining metabolic reprogramming through an epigenetic lens, the authors identify that IGF2BP3 recognizes m6A modifications on mRNAs encoding serine synthesis pathway enzymes and stabilizes these mRNAs, allowing for increased serine biosynthesis. The authors focus on the importance of serine biosynthesis, and the regulation of this serine synthesis pathway via m6A modifications to mRNAs encoding key enzymes (i.e. PHGDH, PSAT1) involved in this pathway. In this manuscript, the authors make the following claims:

- (1) Both extracellular serine and serine biosynthesis are necessary for AML cell growth.
- (2) IGF2BP3 and METTL14 are important in regulating serine metabolism, and loss of either sensitizes AML cell to serine deprivation-induced growth arrest.
- (3) IGF2BP3 regulates de novo serine biosynthesis, and that IGF2BP3 is utilized in AML cells to increase serine biosynthesis.
- (4) IGF2BP3 regulates serine biosynthesis by recognizing m6A modifications of mRNAs encoding PHGDH and PSAT1 and stabilizing these transcripts/increasing their expression, ultimately leading to increased serine production.
- (5) IGF2BP3 knockdown inhibits leukemogenesis in vivo.
- (6) IGF2BP3 is crucial for the maintenance of AML LSCs/LICs.
- (7) IGF2BP3 is dispensable for normal hematopoiesis.
- (8) Knockdown of IGF2BP3 in combination with serine deprivation inhibits primary AML cell growth and improves survival of mice.

This manuscript is well written and conveys important findings about a novel regulatory pathway for serine metabolism in AML. Strong points in the methodology include the use of an auxin-inducible degree system, the genetic knockout mouse model, the metabolic flux experiments, and direct characterization of IGFBP3 binding to its target mRNAs using RIP-seq. However, loss of IGFBP3 affects many other important pathways that are known to affect AML survival; therefore, it is unclear whether the effects on AML growth and survival are mainly driven by serine metabolism or other targets. In addition, the experiments are mostly performed in selected models that may not be representative of a general effect in AML.

#### Major concerns:

1) The findings are heavily based on MLL-rearranged models (human cell lines and mouse syngeneic), which have the highest expression of IGF2BP3 (Weng et al. Cancer Cell 2022). Whether loss of IGF2BP3 similarly affects AML growth and serine metabolism across most cases of AML, as is implied in the title, is unclear. Both findings should be validated in additional AML models representative of the genetic diversity of the disease before making a general claim. The experiments performed using primary AML samples may alleviate this concern, however they are lacking details regarding the genetic subtype, number of cases, etc.

2) Among the genes identified but not discussed by the authors, BCL2 and ATF4 are known to be involved in survival and the response to limiting amino acids levels, respectively. MYC and glutamine uptake were previously reported as IGF2BP3 targets (36306790). IGF2BP3 also enhances the stability of an apoptotic inhibitor (RCC2) (PMID: 35217832). These targets and others could potentially explain the effect seen on leukemogenesis. Therefore, the claim that augmentation of serine biosynthesis by IGF2BP3 drives stemness is not completely proven; rather, IGF2BP3 is important for leukemogenesis and IGF2BP3 activity promotes serine metabolism in the MLL/AF9 model. I would have liked to see that overexpression of Igf2bp3 confers better engraftment and stemness than control MA9-transduced HSPCs, and that this effect is abrogated by perturbation of serine synthesis.

3) It does not appear that the specificity of the shRNAs against IGF2BP3 was validated using rescue overexpression experiments. As a more general comment, it is unclear why some experiments in the murine MLL/AF9 model were performed with shRNA knockdown (i.e. Fig 5K,L, Fig 6F,G) when knock-out mouse models are available (genetically cleaner).

4) The claims of altered PHGDH and PSAT protein levels (Fig 3A-D) should be backed by quantification and statistical analysis.

5) line 310: "In line with the observed changes in the L-GMP population, KO of Igf2bp3 significantly decreased LSCs/LICs frequency (Fig 5J)". It is generally recognized that LSCs are functionally defined through in vivo assays. These should be performed, otherwise an alternate terminology should be used, i.e. "in vitro clonogenic potential".

6) line 322: "deletion of Igf2bp3 does not affect HSC and multilineage development during normal hematopoiesis". Similar to 3), the gold standard definition of HSC is the ability to repopulate an irradiated recipient. Competitive bone marrow transplantation should be performed to claim that there is no impact on HSC numbers; otherwise this claim should be limited to "normal hematopoietic differentiation at steady state".

7) The serine synthesis pathway has other key enzymes that are involved. Is it possible that decreased expression/activity of PHGDH could be inhibiting cell growth through a serine-independent pathway? To strengthen their argument, the authors

should perform similar knockdown experiments with PSAT1 and PSPH to conclude that the SSP is equally crucial to AML survival/growth as extracellular serine.

8) In Figure S1D, the authors show that the small molecule inhibitor of PHGDG reduces AML cell growth. They use a dose of 10  $\mu$ M of NCT-503. However, the authors do not contextualize the relevance of this specific dose. I would encourage the authors to add in a dose-response curve for NCT-503 for each cell line. It is necessary to rule out that the dose chosen does not induce cell death and/or off-target effects that could be inhibiting AML cell growth.

Minor concerns:

In AML the RNA-binding protein YBX1, which binds to methylated (m6A) transcripts via IGF2BP-family of proteins (PMID: 33763698), impairs the translation of oncogenic transcripts (MYC, BCL2 and MCL1) and decreases growth and clonogenic potential of AML cells lines and patient samples (PMID: 34465866). A comment on this work needs to be incorporated in the discussion. Would YBX1 recapitulate IGF2BPs loss? Is YBX1, IGF2BP2 or other m6A binding proteins among the CRISPR screen hits?

The bibliography review related to the regulation of serine metabolism in AML is a bit superficial. For example, ATF4 (a hit in the screen) is known to regulate serine metabolism transcriptionally (33436370) - could this be a confounder? The authors do not refer to possible mechanisms hindered by PHGDH loss in limited serine/glycine availability (mTOR signaling downregulation, enhanced ROS levels, reduced protein synthesis)

There is little mention of the possible role of IGF2BP3 in the translation of PHGDH and PSAT1 mRNAs, rather than stability. I believe that this data should be available from Weng et al. Cancer Cell 2022.

For all growth curves, please add error bars and specify the sample size.

The incorporation of C13-labeled glucose into the TCA cycle and connected metabolic pathways (i.e. highlighted in Fig 2C) should be reported in the supplementary. This would be especially helpful to understand if there is a specific effect on serine synthesis or other synthetic pathways are likewise affected.

Line 132. Remove "s" from Fig.

The authors do not reference Figure 1G in the text.

Line 143. Define acronym MOI.

Line 160. Provide rationale for secondary IGF2BP3 inhibition strategy. Why was mRNA knockdown via sgRNA/shRNA not enough to demonstrate that IGF2BP3 inhibition suppressed AML survival/growth?

Line 182. No need to elaborate that citric acid cycle is also TCA cycle. Just write tricarboxylic acid cycle. No need for "TCA" abbreviation as TCA is only mentioned one more time in the text.

Line 184. Remove "considering the facts" and restructure this sentence. Does not flow.

Line 185. No need to provide acronym for alpha-ketoglutarate – never mentioned again in text.

Line 191. Missing space before "Interestingly, ..."

Line 198. Is this the first report of m6A regulators controlling biosynthesis of an amino acid?

co-review by Francois Mercier and an Early Career Researcher

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I think you did a remarkable job in revising the manuscript accordingly.

Reviewer #2

(Remarks to the Author)

The authors did a good job revising this manuscript. I have no further comments.

Reviewer #3

(Remarks to the Author)

General comments

The authors have done a great job at improving the manuscript and addressing my concerns. Below are some remaining points that need to be addressed:

1. In addition to cell cycle arrest and apoptosis mediated by knockdown of IGF2BP3 in the MLL/AF9 models, could the authors comment if there is an effect on differentiation? The Wright-Giemsa stains from the BMT recipient mice (Fig 4C,N) exhibit morphological changes suggesting differentiation, but it is unclear if we are looking at sorted leukemic cells or total bone marrow (i.e. possibly including normal cells) - it would be very helpful to know how the cells shown in the panel "BM" were isolated. For this assay, ideally, sorted leukemic cells should be compared between conditions. Likewise, in the flow cytometric analysis in Fig S5C, it is unclear where the gating for CD45.1+ cells (i.e. transplanted AML) is done; so it is unclear if this is the immunophenotypic pattern of total bone marrow or leukemic cells.
2. The rescue overexpression experiment (provided as figure RL-24 in the rebuttal) and the quantification of immunoblotting (RL-25) both address important technical concerns and should be included in the supplementary figures.
3. Could the authors add a commentary on the relationship between IGF2BP3 expression and cell growth/survival among AML cells? (specifically because they chose two cell lines (MOLM14 & NB4) which have relatively higher expression, and two cell lines (MM6 and U937) which have relatively lower expression for their assays).

Minor comments:

Line 36: add "the" in front of mechanism

Line 92: change "could" to "can"

Line 107: add "a" in front of synergistic effect

Figure S1O: label that it is MOLM13

Line 191: "serine metabolism was [noted] because it..."

Line 191-194: please rework this sentence. Very long and confusing.

Line 225: "an enzyme [that] plays an..."

Line 263: rescue instead of "rescued"

Line 297: has instead of "have"

Line 298: Was it ectopic expression of human genes (ATF4, PHGDH, PSAT1) or mouse (Atf4, Phgdh, and Psat1)?

Line 313: is instead of "was"

Line 350-352: please correct this sentence. Igf2pb3 is mouse, IGF2PB3 is human.

Line 354: what is the proof for "stress hematopoiesis"? I would recommend adding stress of transplantation.

Line 406: fix font discrepancy

Line 408: replace "is" with "the"

co-review by Francois Mercier and an Early Career Researcher

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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**SUMMARY:** We are very grateful to the Reviewers for their positive appraisal of our manuscript and for their thoughtful and constructive comments and suggestions, which have significantly improved our manuscript. We also sincerely appreciate the editor's careful consideration of our manuscript and his very helpful suggestions. We have conducted a large set of additional experiments and data analyses, and have carefully revised our manuscript to address all the concerns/critiques raised by the Reviewers accordingly. The following is a summary of the major new experiments and data analyses that we have conducted during the revision:

1. An additional AML cell line (NB4) has been added to confirm the roles and mechanism of IGF2BP3 in regulating AML cell proliferation, survival, and response to serine/glycine (SG) deprivation. Moreover, FLT3-ITD/NPM1-mut and PML-RARa models have been used for CFA assays in addition to the original MLL-AF9 model. For primary patient samples, we used one with FLT3-TKD and added one with MLL-rearrangement in the revision. The addition of these AML models has confirmed the critical roles of IGF2BP3 in AML, regardless of the subtype.
2. *ATF4* has been included as a target gene of IGF2BP3. In addition to *PHGDH* and *PSAT1*, we found that *ATF4* is also a direct target of IGF2BP3 that partially mediates the effect of IGF2BP3 in serine metabolism. Interestingly, ATF4 was reported as a transcription factor that regulates the expression of SSP genes. Therefore, IGF2BP3 directly regulates *ATF4*, *PHGDH*, and *PSAT1* expression via m<sup>6</sup>A-dependent post-transcriptional regulation, while *PHGDH* and *PSAT1* are also transcriptionally regulated by ATF4. Our rescue experiments strongly suggest that the serine synthesis pathway plays a critical role in IGF2BP3-mediated AML survival and stemness.
3. Cell proliferation and/or apoptosis have been examined in all human AML cell lines, as well as in primary patient cells with FLT3-TKD mutation or MLL-rearrangement.
4. For the metabolic tracing, we reanalyzed our data and presented the results according to the suggestions of the reviewers. The data show that IGF2BP3 KD resulted in reduced incorporation of C13-glucose into serine and its downstream metabolites, including nucleotides, GSH, and the intermediates of the TCA cycle.

5. We added data from competitive bone marrow transplantation to show that Igf2bp3 KO does not affect normal HSC and progenitor numbers and function upon irradiation-induced stress. These data, together with the data presented in our first submission, indicate that Igf2bp3 is dispensable for normal hematopoiesis either in the steady state or under stress. This is reasonable considering that Igf2bp3 and its SSP-related target genes are expressed at an extremely low level in the whole blood system compared to other tissues and organs.

All the text in our revised manuscript that we have changed or added is marked in **RED**. The deleted text has been removed entirely from the manuscript. We hope the revised manuscript would fulfill the requirements of the Reviewers as well as the editor and will be suitable for publication in *Nature Communications*.

**The following are our point-by-point responses to the comments/suggestions from the reviewers:**

**1. Responses to the comments of Reviewer #1:**

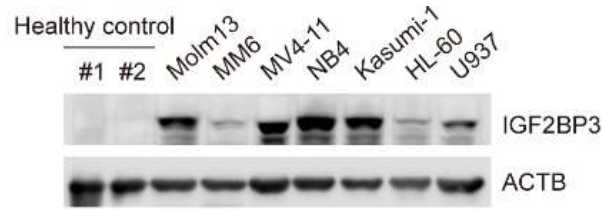
**General comments from Reviewer #1:** *The authors present a very nice study on how M6A regulatory protein IGF2BP3 is involved in leukemogenesis, which may in part be regulated via control of SPP enzymes PHGDH and PSAT1. Novelty of this research is very high. Yet, some critical points are missed out on or over interpreted, as I will indicate and further explain below.*

**Response:** We sincerely appreciate your positive appraisal of our manuscript and for the constructive comments/suggestions.

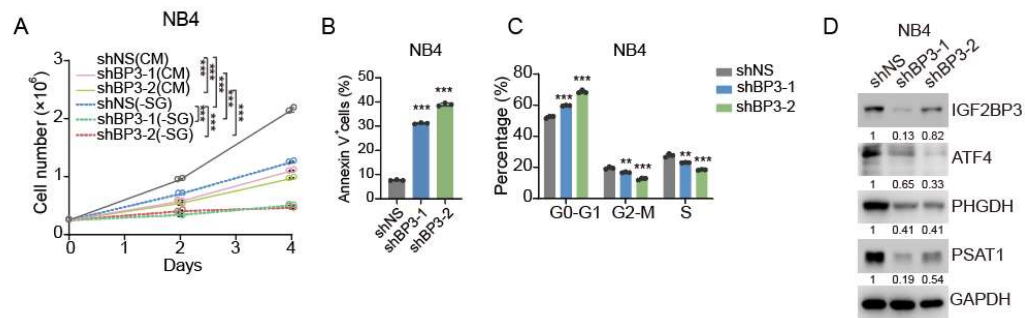
**Comment 1 from Reviewer #1:** *The models: It is unclear to me why these AML models were chosen. They are quite uncommon AML cell models. Especially with respect to their metabolic dependency. One representing a FLT3-ITD MOLM13 model, where FLT3-ITD is known to induce glycolysis differently from FLT3 WT cells. U937 cells are*

*lymphoma cells from pleural effusion, which do not represent an AML. Than the MM6 cells with a MLL-AF9 fusion like the mouse model. MLL-rearranged AML are a special type of AMLs. The MLL-rearranged AMLs are very dependent on RAS pathway activation, therefore, often representing RAS mutations a bit more frequently than other AML subtypes.*

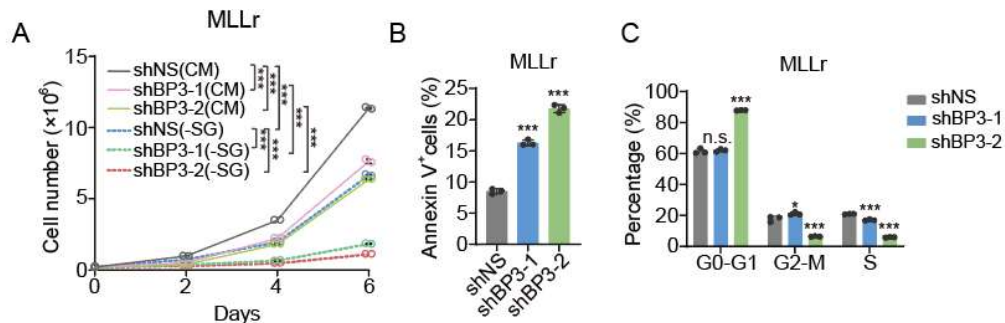
**Response:** We apologize for not describing this clearly in the first version of our manuscript. In fact, we performed a Western blotting analysis of the IGF2BP3 protein in multiple AML cells lines, such as those with MLL fusions (Molm13, MM6, and MV4-11), PML-RARa (NB4), and AML1-ETO (Kasumi-1), along with two cord blood (CB) mononuclear cell (MNC) samples from healthy donors as healthy controls. Among them, Molm13 and MV4-11 cell lines also harbor FLT3-ITD mutations. It should be noted that although the U937 cell line was originally established from the pleural effusion of a 37-year-old man with generalized diffuse histiocytic lymphoma, it exhibits many characteristics of monocytes<sup>1-3</sup> and is often used in the study of AML, therefore we also included it in our analysis. Compared to the normal MNCs, all the cell lines tested exhibited higher expression of IGF2BP3 (RL-Fig.1, incorporated into the revision as Fig S1K), suggesting that overexpression of IGF2BP3 is a common event in AML. We therefore selected Molm13 to represent cell line with relatively high level of IGF2BP3, while U937 and MM6 to represent cell lines with relatively low levels of IGF2BP3, for further studies. To better support our conclusion, we also included the NB4 cell line, in which IGF2BP3 is highly expressed, into our revision (RL-Fig.2, also see Figs 1H, 3A, S3E, and S3F in the revision). Regarding to our primary AML samples, we initially used patient sample with FLT3-TKD mutation, and now added another patient sample with MLL-rearrangement in the revision (RL-Fig.3, also see Figs S3E, S3G, and S7C). Furthermore, in addition to the MA9-transformed mouse hematopoietic stem/progenitor cell (HSPC) model, we have also included the FLT3-ITD mouse bone marrow (BM) cells and the PML-RARa-transformed mouse HSPC models in our CFA assays (RL-Fig.4, also see Figs 4E and 4F in the revision) during the revision. Our data collectively suggest that IGF2BP3 is highly expressed in AML and is critical for AML development and maintenance.



**RL-Fig.1** Western blotting of IGF2BP3 in CB MNCs from healthy donors as well as in cell lines.

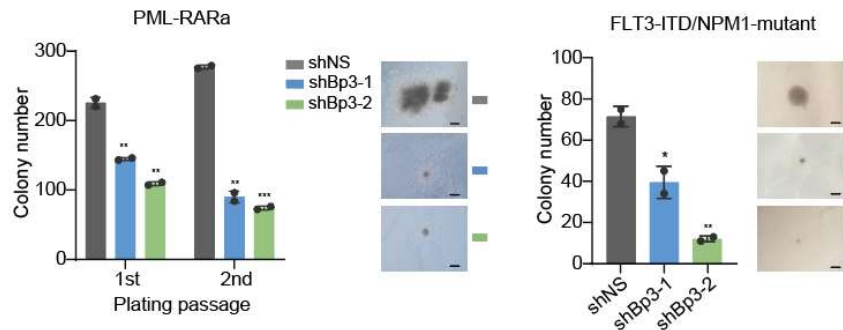


**RL-Fig.2** Phenotypes of knocking down IGF2BP3 in NB4 cells. (A) Growth curves of control or IGF2BP3 KD NB4 cells in CM or -SG medium. (B) Percentages of Annexin V<sup>+</sup> apoptotic cells in NB4 cells with or without *IGF2BP3* knockdown as detected by flow cytometry. (C) Percentages of different cell cycle phases in NB4 cells with or without *IGF2BP3* knockdown as detected by flow cytometry. (D) Western blot showing expression change of ATF4, PHGDH and PSAT1 after *IGF2BP3* KD in NB4 cells. GAPDH was used as a loading control.



**RL-Fig.3** Phenotypes of knocking down IGF2BP3 in AML PDX cells with MLLr-rearrangement (MLLr). (A) Growth curves of AML PDX cells with MLLr upon *IGF2BP3* KD in CM or -SG medium. (B) Percentages of Annexin V<sup>+</sup> apoptotic cells in AML PDX cells upon *IGF2BP3* KD in CM or -SG medium. (C) Percentages of different cell cycle phases in AML PDX cells with MLLr upon *IGF2BP3* KD in CM or -SG medium.

*IGF2BP3* knockdown as detected by flow cytometry. (C) Percentages of different cell cycle phases in AML PDX cells upon *IGF2BP3* knockdown as detected by flow cytometry.



**RL-Fig.4** Knockdown of *IGF2BP3* inhibits colony formation of mouse HSPCs immortalized by PML-RARa (left) or BM cells from FLT3-ITD/NPM1-mut mice (right).

**Comment 2 from Reviewer #1:** *In general, many genes pop out of the epigenetic screen from which M6A regulatory proteins were cherry picked. What about the other genes? NRAS is part of the top30 genes SG dependent scores, so at least define the difference between NRAS WT and NRAS mutant AML with respect to IGF2BP3 dependency. Maybe comparing these would be more clear with respect to rescue experiments you represent in the paper, where you only see maximum of 50% rescue, appointing that a lot more is going on besides SPP pathway regulation. Serine levels are also only slightly affected by your IGF2BP3 KO guide RNAs. Plus ATF4 is also in your list of IGF2BP3 regulated, so if the SPP pathway regulation partially due to IGF2BP3 regulation and in part indirectly through ATF4 regulation or maybe even only through ATF4 regulation? This should be clarified. Also SIRT4 is in your screen, which is affecting glutamine metabolism and indirectly SPP metabolism. What are NRF2, MYC, ATF4 levels in your models/experiments? Is it all mediated via these transcriptional regulators rather than direct IGF2BP3 effects?*

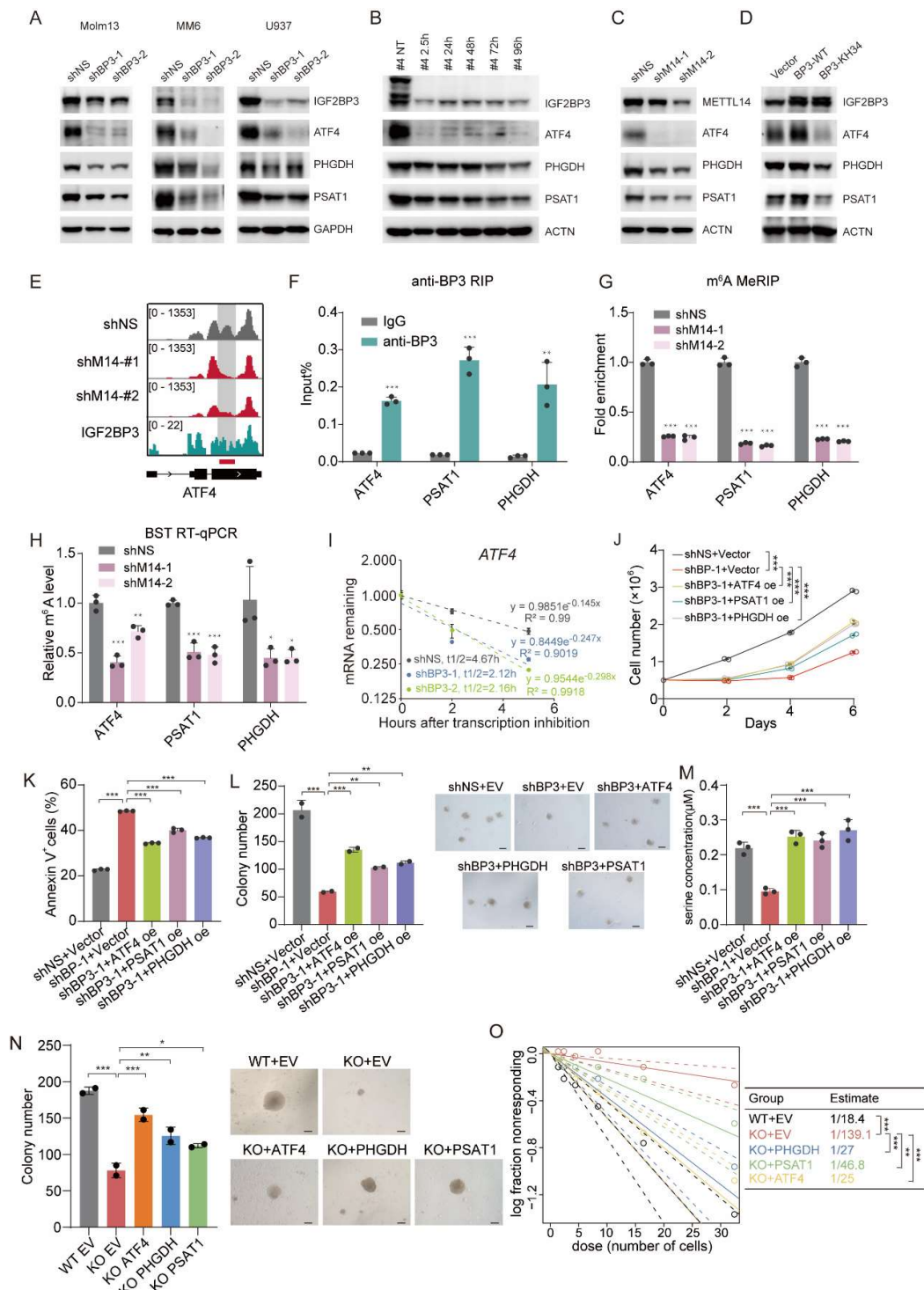
**Response:** Thank you for your suggestions. A major reason that we focused on the m<sup>6</sup>A pathway after screening is that both the m<sup>6</sup>A reader (*IGF2BP3*) and writer (*METTL14*) were on the top list of top candidates whose loss sensitized AML cells to serine/glycine deprivation, indicating that the m<sup>6</sup>A pathway plays a role in serine metabolism.

Nonetheless, we agree with you that other genes from our screen may also play a role in AML. Particularly, for NRAS, we have followed your suggestion to analyze the dependency of IGF2BP3 in NRAS WT and mutant AML using the data from Depmap (<https://depmap.org/portal/>). However, the CERES scores of IGF2BP3 in AML cell lines with NRAS mutation display no significant difference ( $p = 0.4523$ ) to those with WT NRAS (RL-Fig.5A). In addition, no significant difference was observed for the mRNA levels of IGF2BP3 and its downstream targets between NRAS WT and mutant AML patients (RL-Fig.5B-5C). These data suggest that the dependency of AML on IGF2BP3 is not determined by the status of the NRAS gene. SIRT4 also shows up in our screen, and we have discussed its potential role in the Discussion section.

[Figure redacted]

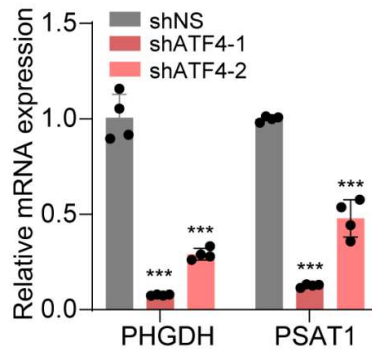
**RL-Fig.5** The dependency of AML on IGF2BP3 is regardless of NRAS mutations. (A) Correlated CERES scores of IGF2BP3 in AML cell lines based on the presence or absence of NRAS mutations. (B, C) Relative mRNA expression levels of IGF2BP3 (B) and its target genes (C), including ATF4, PSAT1 and PHGDH, in AML patients with or without NRAS mutation.

In terms of the target genes, we actually also examined *ATF4* along with *PHGDH* and *PSAT1*, and have found that *ATF4* is also a direct target of IGF2BP3 that partially mediates the effect of IGF2BP3 in serine metabolism (RL-Fig.6, also see Figs 3A-3M, S3D, S3M, 4O, S4H-S4L, 5M and 5N in the revision). Interestingly, ATF4 was reported as a transcription factor that regulates the expression of SSP genes<sup>4,5</sup>, which was also confirmed by us (RL-Fig.7, also see Fig. S3C). Therefore, IGF2BP3 directly and post-transcriptionally regulates *ATF4*, *PHGDH*, and *PSAT1* expression as an m<sup>6</sup>A reader, while *PHGDH* and *PSAT1* are also transcriptionally regulated by ATF4. We have added these results into the revision.



**RL-Fig.6** ATF4 is a functional target of IGF2BP3. (A) Western blot showing expression change of ATF4, PHGDH, and PSAT1 after *IGF2BP3* KD in various AML cell lines. (B) Western blot showing expression change of ATF4, PHGDH, and PSAT1 in U937 BP3-mAID cells after treatment with 5'Ph-IAA for the indicated time periods. (C) Western blot showing

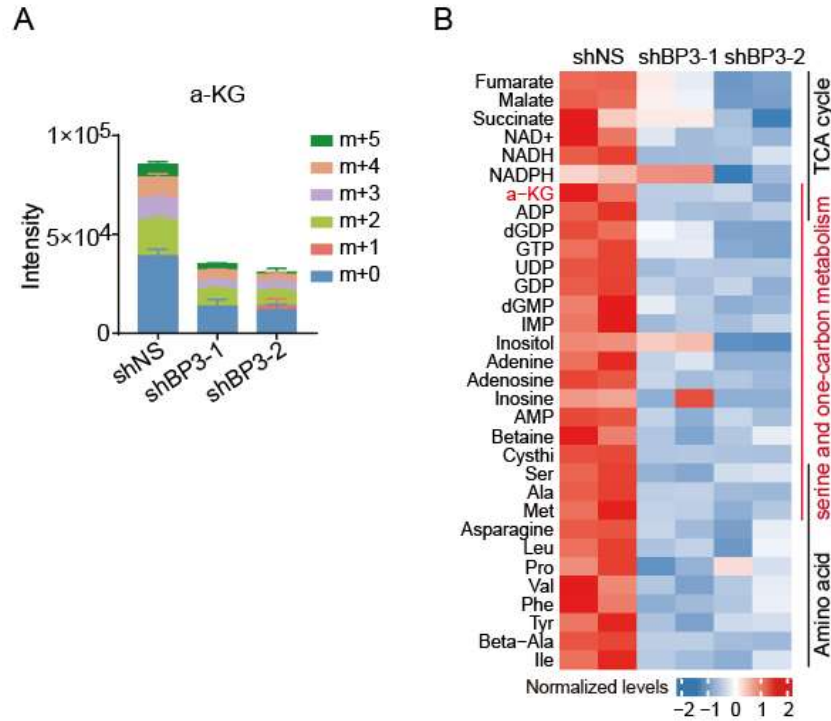
expression change of ATF4, PHGDH, and PSAT1 in Molm13 cells after *METTL14* KD. (D) Western blot showing expression change of ATF4, PHGDH, and PSAT1 in Molm13 cells after overexpression of wild-type (BP3-WT) or KH3-4 mutated (BP3-KH34) IGF2BP3. (E) IGV tracks showing the distribution of m<sup>6</sup>A (GEO: GSE97408) or IGF2BP3 binding sites (GEO: GSE90639) in the mRNA transcripts of ATF4. Grey shadow depicts high-confidence m<sup>6</sup>A regions for qPCR validation in (F) and (G). (F) RIP assays using an IGF2BP3 antibody were performed in Molm13 cells, followed by qPCR to detect direct binding of IGF2BP3 to *ATF4*, *PHGDH*, and *PSAT1* transcripts at regions indicated in (E). (G) MeRIP-qPCR was performed in control and *METTL14* KD Molm13 cells to detect the change of m<sup>6</sup>A abundance at indicated regions of *ATF4*, *PHGDH*, and *PSAT1* transcripts. (H) Bst DNA polymerase-mediated cDNA extension and qPCR assays to evaluate relative m<sup>6</sup>A abundance changes at specific sites within *PHGDH* and *PSAT1* transcripts in control and *METTL14* KD Molm13 cells. (I) Control and *IGF2BP3* KD Molm13 cells were treated with actinomycin D for indicated time periods and the RNA level of ATF4 was examined by qPCR. 18S rRNA was used as a loading control. The mRNA half-life ( $t_{1/2}$ ) was calculated and shown. (J, K) Live cell counting (J) and cytometric analysis of Annexin V<sup>+</sup> apoptotic cells (K) in Molm13 cells upon *IGF2BP3* KD and overexpression of IGF2BP3 target genes, including ATF4. EV, empty vector. (L, M) Molm13 cells were co-transduced with control or *IGF2BP3* shRNAs along with target gene overexpression vectors and subjected to colony-forming assays (L) and serine level detection (M). (N) HSPCs from WT or *Igf2bp3* KO mice were co-transduced with *MA9* and ATF4-, PHGDH-, or PSAT1-overexpressing viruses and seeded for CFA. (O) HSPCs collected from *Igf2bp3* WT and HO mice were co-transduced with *MA9* and target gene-overexpressing viruses and selected in methylcellulose medium with G418 and puromycin for 7 days before seeded for LDA assays.



**RL-Fig.7** ATF4 transcriptionally regulates *PHGDH* and *PSAT1* expression. The relative mRNA expression of *PHGDH* and *PSAT1* after *ATF4* knockdown in Molm13 cells was detected by qPCR.

**Comment 3 from Reviewer #1:** *In figure 2B, AKG is listed in your TCA but also comes from SPP, form metabolic tracing experiments it should be clear what is coming from where. Furthermore, αKG is important for RNA methylation, and might explain your phenotype rather than SPP pathway beyond PSAT1?*

**Response:** Thank you for your suggestion. We have checked our metabolic tracing data and found that approximately 40% of αKG was labeled with C<sub>13</sub> (RL-Fig.8, also see Fig S2A in the revision), suggesting that they were produced during serine synthesis. We also rearranged the original Fig 2B (RL-Fig.8, also see Fig 2B in the revision) following your suggestion. As mentioned by you, αKG is a substrate for demethylases and might explain part of the IGF2BP3 effect in addition to the SSP pathway. We have included this in the Discussion section.

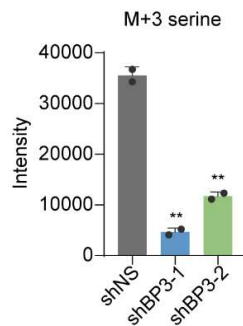


**RL-Fig.8** IGF2BP3 regulates serine biosynthesis and  $\alpha$ KG levels. (A) The total levels and isotopolog distribution ( $M + n$ ,  $n$  refers to numbers of  $C_{13}$ ) of  $\alpha$ KG measured by LC-MS in Molm13 cells transduced with IGF2BP3 shRNAs or shNS and grown in medium containing [U- $^{13}C_6$ ]-glucose. (B) Heatmaps showing levels of representative metabolites after IGF2BP3 KD in Molm13 cells.

**Comment 4 from Reviewer #1:** *What I also don't understand is your labeling experiment. If cellular serine is made from glucose, you would obtain M+3 serine. I have never observed these high levels of M+1 serine from labeled  $^{13}C$ -glucose. Where do these come from?*

**Response:** We apologized for the mistake in displaying labeled serine. After carefully examining our original LC-MS data, we found that an incorrect peak was assigned to serine in the negative mode of detection, possibly attributed to the low detection rate of serine under this condition. Therefore, we switched to the data in the positive mode and obtained the correct result (RL-Fig.9, also see Fig. 2E in the revision). As you mentioned, serine made from U $^{13}C$ -glucose should be M+3, thus we only show the

M+3 fraction of serine to avoid any confusion. The new result indicates that serine synthesis from glucose is remarkably inhibited upon IGF2BP3 knockdown.

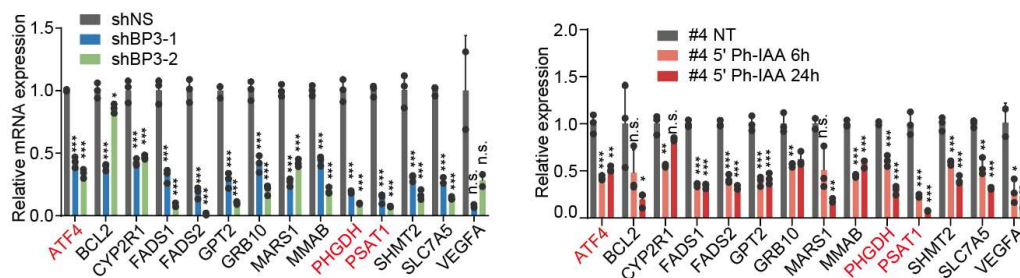


**RL-Fig.9** Peak area intensity of M+3 serine measured by LC-MS in Molm13 cells transduced with IGF2BP3 shRNAs or shNS and grown in medium containing [U-13C6]-glucose.

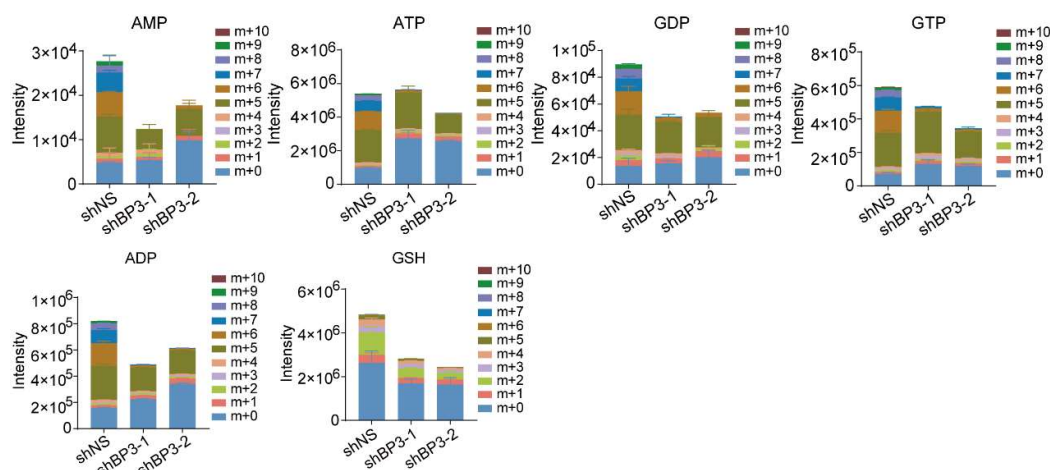
**Comment 5 from Reviewer #1:** *Besides PHGDH and PSAT1 also SHMT2 from the SPP pathway is included as IGF2BP3 hit, why is it excluded? This enzyme converts serine to glycine for further processing toward GSH and nucleotides. To provide a clear overview, all enzymes should be checked and presented if you want to claim these effects are specific for your enzymes of interest. Getting to my next question; What happens downstream of the SPP pathway, what are they using serine for? What is controlled by IGF2BP3 mediated enzyme expression?*

**Response:** As you mentioned, SHMT2 plays an important role in converting serine to glycine and supplying carbon to the one-carbon metabolism. We did check the expression change of *SHMT2* upon IGF2BP3 knockdown or acute depletion. Similar to *PHGDH*, *PSAT1* and *ATF4*, *SHMT2* exhibited a remarkable decrease when IGF2BP3 was depleted (RL-Fig.10, also see Figs S2F and S2G in the revision), suggesting that it is also a potential target gene of IGF2BP3. As we focused on the serine biosynthesis but not catabolism, we did not include *SHMT2* into further mechanistic studies. Nonetheless, isotopolog distribution of nucleotides and GSH confirmed that the synthesis of the downstream metabolites in the one carbon metabolism pathway was inhibited when IGF2BP3 was knocked down (RL-Fig.11, also see Figs 2C and S2A in the revision). These results together suggest that IGF2BP3 regulates both serine

synthesis and catabolism to sustain the one-carbon metabolism and energy supply of AML cells. We have discussed this in the revised manuscript now.



**RL-Fig.10** Validation of potential targets of IGF2BP3 in the “L-serine biosynthetic process” GO term by qPCR after IGF2BP3 KD in Molm13 cells (left) or IGF2BP3 degradation in U937 cells (right).



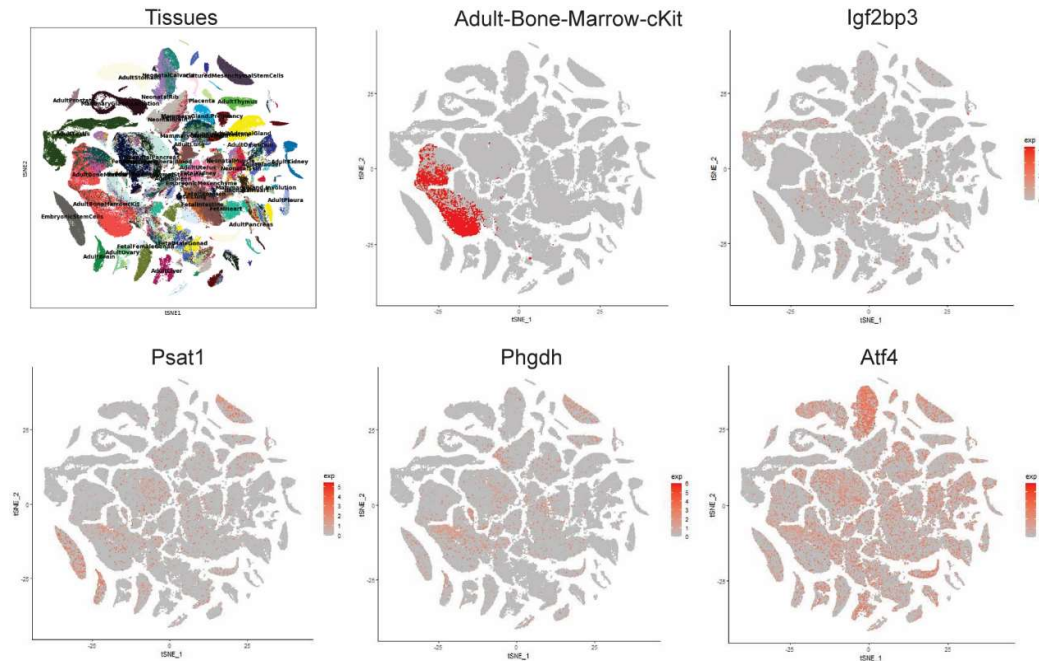
**RL-Fig.11** The total levels and isotopolog distribution (M + n, n refers to numbers of  $^{13}\text{C}$ ) of nucleotides and GSH measured by LC-MS in Molm13 cells transduced with *IGF2BP3* shRNAs or shNS and grown in medium containing  $[\text{U-}^{13}\text{C}_6]$ -glucose.

**Comment 6 from Reviewer #1:** *I am still puzzled why BP3 KO mice show so little effects. If IGF2BP3 is controlling SSP genes, these mice should present neurologic phenotypes and diseases. So something seems overlooked.*

**Response:** IGF2BP3 and other proteins from the same family have been recognized as oncofetal proteins that are highly expressed in embryos but the expression levels decrease to very low in adult tissues<sup>6-8</sup>. Indeed, IGF2BP3 and its SSP-related target

genes are expressed at low levels across various tissues and organs, particularly in whole blood of adult humans, according to data from The Adult Genotype Tissue Expression (GTEx) Project (RL-Fig.12, also see Fig S6 in the revision). In addition, the expression levels of Igf2bp3 and its SSP-related target genes were low in mouse BM c-kit<sup>+</sup> cells (RL-Fig.13), which represent hematopoietic stem/progenitors cells in mice. These data suggest that in normal adult tissues or organs, the post-transcriptional regulation of target genes by IGF2BP3 is relatively weak, and therefore, the impact of its knockout would likely be very limited. Together, these results may explain the marginal impact of IGF2BP3 knockout on normal blood development. Nonetheless, it should be noted that our study focused solely on the hematopoietic system. Whether knockout of Igf2bp3 affects the function of other organs, such as the nervous system, may require long-term monitoring.





**RL-Fig.13** Expression of Igf2bp3 and its SSP-related target genes in mouse BM c-kit<sup>+</sup> cells from scRNA-seq data (<https://bis.zju.edu.cn/MCA/index.html>).

## 2. Responses to the comments of Reviewer #2:

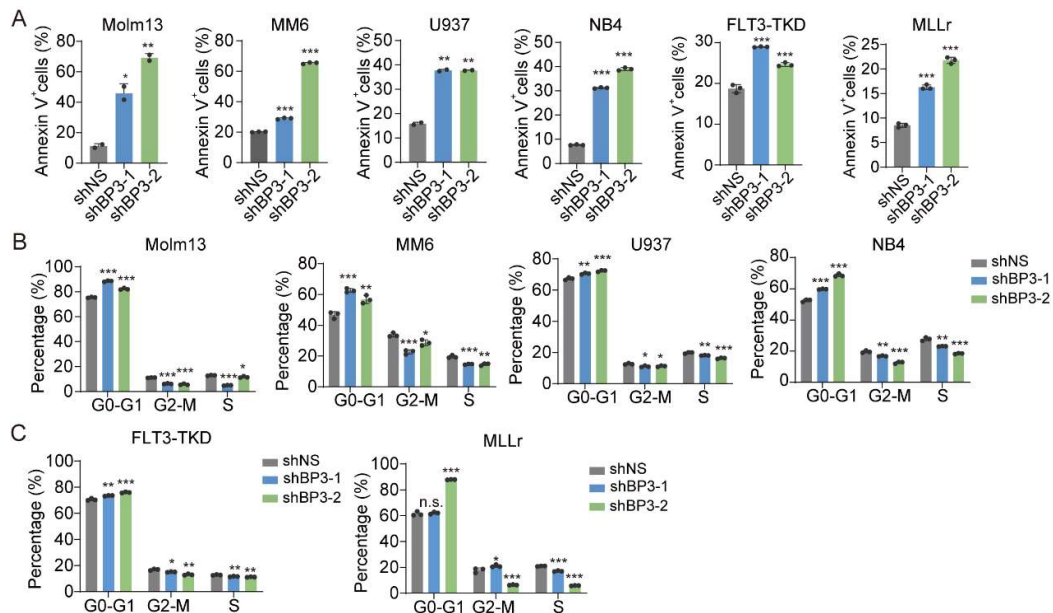
**General comments from Reviewer #2:** *Huang et al present a compelling case that IGF2BP3 is a potential target in AML, especially in combination with SG-deprivation in vitro and in vivo, however, the manuscript often contains biased experiments, often inappropriate controls to prove their hypothesis. Moreover, the title “Augment of Serine Biosynthesis by m6A Modification Drives Leukemogenesis and Stemness as a Metabolic Vulnerability in AML” is inappropriate for this publication given that the study focuses primarily on IGF2BP3, and at points METTL14, but does not consider other fundamental players in m6A biology.*

*To consider this manuscript for publication, I suggest a change in title, as well as the following.*

**Response:** Thank you for your constructive comments/suggestions. We have changed the title into “Augment of Serine Biosynthesis by IGF2BP3 Drives Leukemogenesis and Stemness as a Metabolic Vulnerability in AML” in the revised manuscript.

**Comment 1 from Reviewer #2:** *In the majority of the in vitro experiments, the authors show only a loss of proliferation, but do not show whether this is caused by cell cycle arrest, differentiation or cell death. This is present throughout the paper and across models (human AML cell lines, murine AML models and patient samples). IGF2BP3 has been reported to cause cell cycle defects in AML, and so this is a significant omission.*

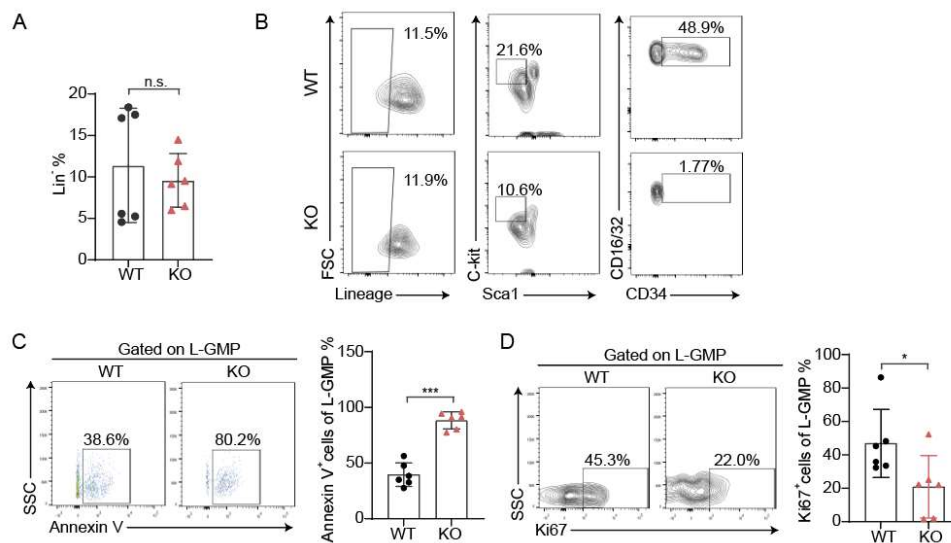
**Response:** In the original Fig. S3D, we have presented data to show that knockdown of IGF2BP3 resulted in increased apoptosis in Molm13, MM6, and U937 cell lines. Following your suggestion, we have included new data showing that knockdown of IGF2BP3 caused an increase in apoptosis and cell cycle arrest in these cells lines as well as in NB4 and AML PDX samples (RL-Fig.14, also see Figs S3E-S3G in the revision).



**RL-Fig.14** Knockdown of IGF2BP3 leads to increased apoptosis and cell cycle arrest. (A) Percentages of Annexin V+ apoptotic cells in various AML cell lines and PDX cells with or without IGF2BP3 knockdown as detected by flow cytometry. (B and C) Percentages of different cell cycle phases in various AML cell lines (B) and PDX cells (C) with or without IGF2BP3 knockdown as detected by flow cytometry.

**Comment 2 from Reviewer #2:** A related point is the L-GMP analyses in Figure 5H/I, where the authors show a loss of L-L-GMPs in the KO mice. Is the loss of IGF2BP3 causing a loss in a leukaemia stem cell marker through apoptosis or cell cycle arrest of these cells, or is it increasing a differentiation marker? There is in fact an increase in Lineage positive markers in the KO mice shown in 5H, can the make-up of the lineage compartment be provided?

**Response:** The percentage of Lin<sup>-</sup> cells seemed to be different between WT and KO in the original Fig 5H. However, when we analyzed the statistic percentage of Lin<sup>-</sup> cells, we found no significant change in the frequency of Lin<sup>-</sup> (CD45.2<sup>+</sup>Lin<sup>-</sup>) cells between groups (RL-Fig.15A). Therefore, we replaced the original Fig 5H with flow cytometric results from other mice that better represent the average levels of Lin<sup>-</sup> cells in the corresponding groups (RL-Fig.15B, also see Fig S5C in the revision). Furthermore, we also analyzed the percentages of apoptotic (Annexin V<sup>+</sup>) and proliferating (Ki67<sup>+</sup>) L-GMPs as suggested, and observed increased apoptosis and reduced proliferation of L-GMPs from the Igf2bp3 KO group (RL-Fig.15C and D, also see Figs 5I, 5J, S5D, and S5E in the revision). These data indicate that Igf2bp3 KO leads to a loss in L-GMP through inducing cell apoptosis and cell cycle arrest of L-GMPs. Specifically, lineage markers include CD3, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), and Ter-119.



**RL-Fig.15** Igf2bp3 KO led to a loss in L-GMPs through causing apoptosis and cell cycle arrest. (A) Statistics of the percentage of Lin<sup>-</sup> (CD45.2<sup>+</sup>Lin<sup>-</sup>) population in BM of the primary BMT recipient mice transplanted with MA9-transduced HSPCs from Igf2bp3 WT and KO mice. (B) Flow cytometry gating strategy for the L-GMP population (c-kit<sup>+</sup>Sca1<sup>-</sup>CD34<sup>+</sup>CD16/32<sup>+</sup>) in BM of the primary BMT recipient mice. (C, D) Flow cytometry gating strategy and statistics of Annexin V<sup>+</sup> (C) or Ki67<sup>+</sup> (D) L-GMP population.

**Comment 3 from Reviewer #2:** *The authors often present selected data from biased approaches to test their hypothesis and reach their conclusions. For example, can the authors explain the reasoning behind using an CRISPR library targeting epigenetic genes only? This library would fail to provide a genome wide analyses of the mechanisms behind SG-deprived AML cells.*

**Response:** Epigenetic regulation has been increasingly shown to be important for various physiological and pathological processes. The aim of this study is to identify epigenetic regulators that are critical in regulating serine metabolism in AML, therefore a CRISPR library targeting epigenetic genes was chosen for the screening.

**Comment 4 from Reviewer #2:** *When it comes to the claim in the title that serine biosynthesis is augmented by m6A, the authors only focus on IGF2BP3 and METTL14. Are other key regulators of the m6A modification, e.g. YTHDFs, FTO, ALKBHs also dropped out in the CRISPR-screen? SIF shows the top genes to drop out, where a number are involved in m6A biology, but many are not. Can the authors provide dependency scores of the other key m6A players?*

**Response:** Thank you for your suggestion. We ranked all the epigenetic regulators in our CRISPR screen library based on their impact on cell dependency to serine/glycine (SG) deprivation. Genes with SG-dependent score increments of less than -0.1 between cell populations were used for further studies and shown in RL-Table 1 (also see Table S1 in the revision). To make it clearer, we added the “SG dependent score increment” information for each gene to show the changes of cell dependence on SG upon cell

passaging. The dependency scores and SG dependent score increments for other m<sup>6</sup>A players were calculated as suggested (RL-Table 2).

**RL-Table 1.** The SG dependent scores of the top 30 hits in the screening

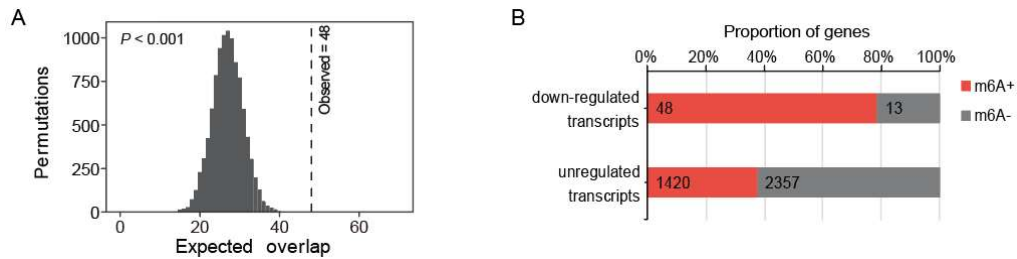
| Gene        | SG dependent scores |       |       | SG dependent score increment |         |
|-------------|---------------------|-------|-------|------------------------------|---------|
|             | P8                  | P12   | P17   | P12-P8                       | P17-P12 |
| HNRNPA2B1   | -0.60               | -0.91 | -1.24 | -0.32                        | -0.32   |
| ORC1        | -0.30               | -0.51 | -0.80 | -0.20                        | -0.29   |
| TUBA1B      | -0.40               | -0.50 | -0.70 | -0.10                        | -0.20   |
| TYW1        | -0.19               | -0.35 | -0.68 | -0.16                        | -0.33   |
| RAD6B       | -0.29               | -0.53 | -0.66 | -0.24                        | -0.12   |
| P300        | -0.27               | -0.46 | -0.66 | -0.18                        | -0.20   |
| IGF2BP3     | -0.29               | -0.50 | -0.63 | -0.21                        | -0.13   |
| TFB2M       | -0.04               | -0.22 | -0.62 | -0.18                        | -0.40   |
| CFP1        | -0.31               | -0.46 | -0.62 | -0.15                        | -0.16   |
| NSUN5P1     | -0.11               | -0.27 | -0.61 | -0.16                        | -0.35   |
| MYB         | -0.38               | -0.51 | -0.61 | -0.13                        | -0.10   |
| RPUSD1      | -0.30               | -0.47 | -0.59 | -0.17                        | -0.12   |
| TAF3        | -0.22               | -0.37 | -0.58 | -0.15                        | -0.21   |
| METTL1      | -0.21               | -0.38 | -0.58 | -0.17                        | -0.20   |
| ALKBH8      | -0.09               | -0.46 | -0.57 | -0.37                        | -0.11   |
| METTL14     | -0.31               | -0.42 | -0.56 | -0.11                        | -0.14   |
| GRIP        | -0.14               | -0.31 | -0.55 | -0.17                        | -0.24   |
| PYGO2       | -0.14               | -0.45 | -0.55 | -0.30                        | -0.10   |
| A1CF        | -0.06               | -0.25 | -0.53 | -0.18                        | -0.29   |
| ZCCHC4      | -0.21               | -0.34 | -0.53 | -0.12                        | -0.19   |
| METTL11B    | -0.24               | -0.38 | -0.50 | -0.13                        | -0.12   |
| IRF7        | -0.11               | -0.30 | -0.49 | -0.20                        | -0.19   |
| SET8/KMT5A  | -0.20               | -0.33 | -0.47 | -0.14                        | -0.14   |
| DUS1L       | -0.17               | -0.34 | -0.47 | -0.18                        | -0.13   |
| GATA2       | -0.09               | -0.27 | -0.46 | -0.18                        | -0.19   |
| RALY        | -0.15               | -0.30 | -0.43 | -0.15                        | -0.13   |
| OSGEP       | -0.13               | -0.23 | -0.35 | -0.10                        | -0.12   |
| NRAS        | -0.04               | -0.18 | -0.34 | -0.14                        | -0.16   |
| PTIP/PAXIP1 | 0.03                | -0.16 | -0.33 | -0.19                        | -0.17   |
| SIRT4       | -0.06               | -0.17 | -0.31 | -0.11                        | -0.14   |

**RL-Table 2.** The SG dependent scores of m<sup>6</sup>A relative genes in the screening

| Gene    | SG dependent scores |       |       | SG dependent score increment |         |
|---------|---------------------|-------|-------|------------------------------|---------|
|         | P8                  | P12   | P17   | P12-P8                       | P17-P12 |
| METTL3  | -0.01               | 0.04  | 0.02  | 0.05                         | -0.02   |
| WTAP    | -0.43               | -0.26 | 0.00  | 0.17                         | 0.26    |
| FTO     | -0.04               | -0.16 | NA    | -0.12                        | NA      |
| ALKBH5  | -0.33               | -0.42 | -0.35 | -0.09                        | 0.08    |
| IGF2BP1 | -0.08               | 0.06  | 0.25  | 0.14                         | 0.19    |
| IGF2BP2 | -0.52               | -0.54 | -0.76 | -0.02                        | -0.22   |
| YTHDF1  | -0.11               | NA    | NA    | NA                           | NA      |
| YTHDF2  | 0.42                | 0.28  | NA    | -0.14                        | NA      |
| YTHDC1  | -0.32               | -0.42 | NA    | -0.11                        | NA      |
| YTHDC2  | -0.06               | NA    | NA    | NA                           | NA      |

**Comment 5 from Reviewer #2:** *Although the proportion of m<sup>6</sup>A modified down-regulated transcripts seems over represented in Figure 2J, since the estimates of the proportion of the genome that is m<sup>6</sup>A-modified varies between 20-60%, can the authors provide a comparison for this? Perhaps transcripts that are unregulated and unchanged?*

**Response:** We agree with you that a statistical test of the proportion of m<sup>6</sup>A-modified down-regulated transcripts is necessary. Therefore, we conducted a permutation test to evaluate whether the overlapping ratio was attributed to randomness. The results showed that the number of overlapping genes (48) was significantly higher than that expected by random distribution (RL-Fig.16A, also see Fig S2D in the revision). Additionally, we found that the proportion of m<sup>6</sup>A modification in unregulated transcripts was 37.6%, whereas that in the down-regulated transcripts was as high as 78.7% (RL-Fig.16B, also see Fig S2E in the revision). These results support that the proportion of m<sup>6</sup>A-modified down-regulated transcripts is significantly overrepresented.



**RL-Fig.16.** (A) The permutation test indicates that the down-regulated transcripts are significantly more likely to m<sup>6</sup>A modified than would be expected by random chance. 10000 permutations were carried out,  $P$  value  $< 0.001$ . (B) Proportion of m<sup>6</sup>A modified transcripts.

**Comment 6 from Reviewer #2:** *Related in Figure 3H, the m6A methylation activity of METTL14 suggests that KD of METTL14 would result in general decrease in m6A abundance. The m6A -qPCR experiments as a result are very focused, and only concentrate on the transcripts involved in SG biosynthesis, and are therefore biased. It is perhaps likely that a vast number of other transcripts would behave in the same way following METTL14 loss. Can the authors provide comparisons with transcripts to prove this is specific to PSAT1 and PHGDH?*

**Response:** As METTL3 and METTL14 are the two major components of the m<sup>6</sup>A methyltransferase complex that deposits m<sup>6</sup>A modifications into RNAs, KD of METTL3 or METTL14 will definitely result in a global decrease of m<sup>6</sup>A abundance on m<sup>6</sup>A-modified transcripts as previously reported by us and other groups<sup>9,10</sup>. Our results in Figure 3H, together with those in Figure 3G, were shown to prove that *PSAT1*, *PHGDH*, and *ATF4* (added in the revision) were regulated by m<sup>6</sup>A modification, but not to prove that they are the only genes regulated by m<sup>6</sup>A.

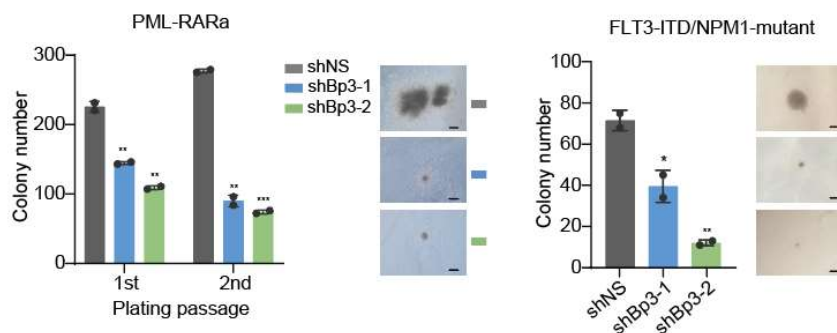
**Comment 7 from Reviewer #2:** *As with Figure 3H, the authors are conducting very focused experiments, and not looking at the global picture. Can the authors consider looking at other targets, for example with SLAM-seq to properly conclude the mechanism proposed.*

**Response:** As stated in the response to your last comment, KD of METTL14 resulted in a global decrease of m<sup>6</sup>A levels in m<sup>6</sup>A-modified mRNAs as reported by us

previously<sup>10</sup>. As we already focused on *PSAT1*, *PHGDH*, and *ATF4* as critical targets of IGF2BP3, we here used BST-RT-qPCR to confirmed that m<sup>6</sup>A abundance at the detected sites on these target transcripts decreased upon METTL14 KD, by referring to our previously published paper<sup>11</sup>. We reason that looking at the global picture of m<sup>6</sup>A changes is not necessary here.

**Comment 8 from Reviewer #2:** *The murine studies in Figure 4 are only conducted using MLL-AF9 driven AML. Is this phenotype seen in other common murine AML models, e.g. NRAS-ETO, FLT3-ITD, PML-RARa?*

**Response:** Thank you for your suggestion. We have included the FLT3-ITD/NPM1-mut and PML-RARa models in our CFA assays (RL-Fig.4, also see Figs 4E and 4F in the revision). The results are in agreement with those of the MLL-AF9 model, demonstrating that IGF2BP3 is critical for leukemic transformation and AML pathogenesis.

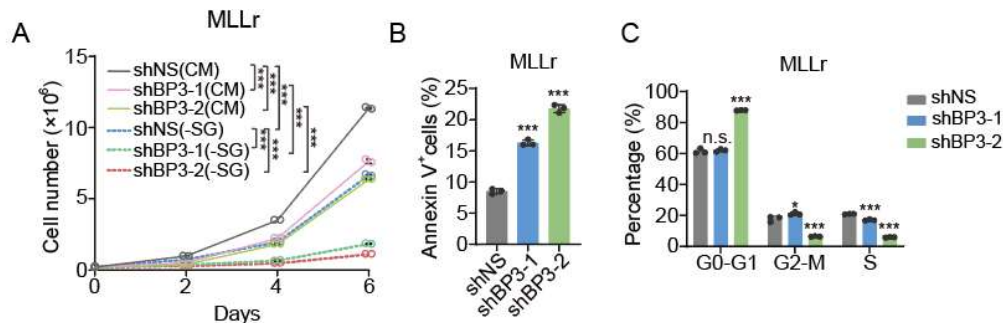


**RL-Fig.4** Knockdown of IGF2BP3 inhibits colony formation of mouse HSPCs immortalized by PML-RARa (left) or BM cells from FLT3-ITD/NPM1-mut mice (right).

**Comment 9 from Reviewer #2:** *Can the authors please provide details of the mutational status of the patient samples used in this experiments? Do the samples used contain similar mutations or are they different and are therefore a broad representation of patient biology.*

**Response:** The patient sample we used in our study harbored a FLT3-TKD mutation. During the revision, we included another patient sample that harbors MLL-rearrangement (RL-Fig.17, also see Figs S3E, S3G, and S7C in the revision), and

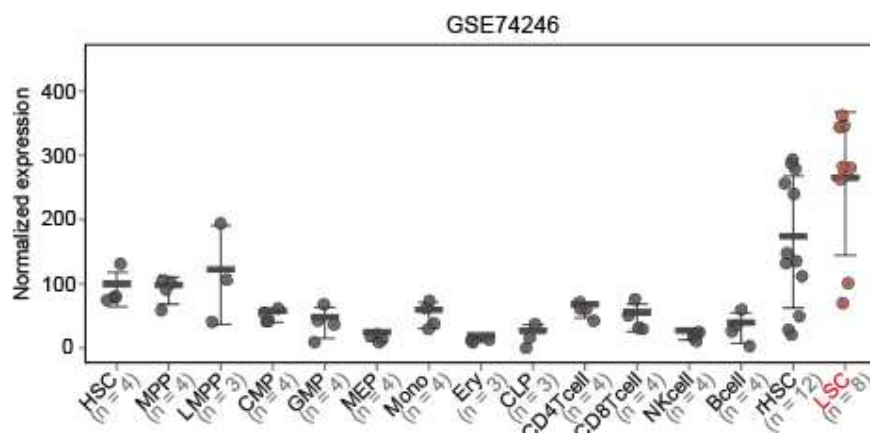
obtained similar results with that from the FLT3-TKD sample. Therefore, our results indicate that IGF2BP3 KD has a broad inhibitory effect on AML patient cells. We have included the mutational status of the patient samples in the corresponding figures.



**RL-Fig.17** IGF2BP3 KD shows inhibitory effects on AML patient cells with MLL-rearrangement (MLLr).

**Comment 10 from Reviewer #2:** Regarding the experiments studying normal haematopoiesis in Figure 5, can the authors provide expression data of IGF2BP3 in the stem and progenitor compartments, and differentiated compartments of normal haematopoiesis.

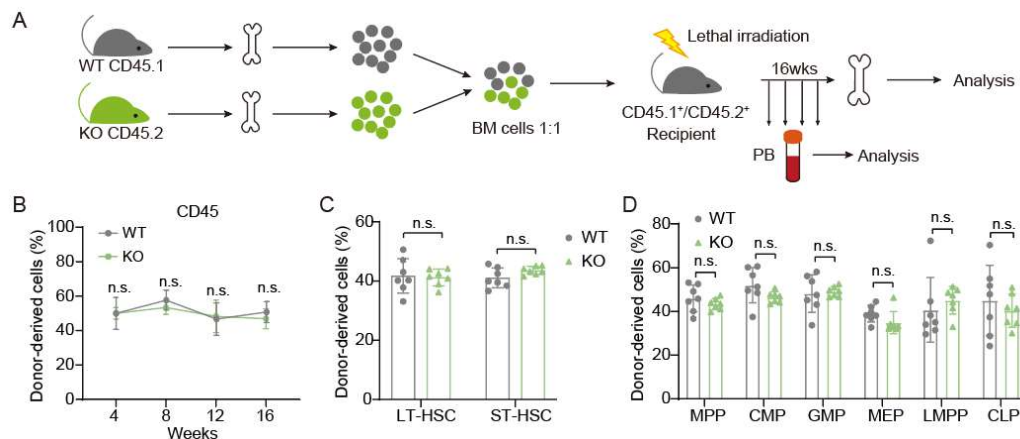
**Response:** Following your suggestion, we have included the expression data of IGF2BP3 in the stem and progenitor compartments, differentiated compartments of normal blood cells, as well as in leukemia stem cells (LSC) (RL-Fig.18, also see Fig S5A in the revision). These data were obtained from GSE74246.



**RL-Fig.18** IGF2BP3 expression in normal hematopoietic compartments and LSC.

**Comment 11 from Reviewer #2:** *Considering the anti-AML phenotype upon IGF2BP3 loss are shown most significantly following transplantation, can the authors provide HSC transplantation data for normal haematopoiesis? This will test whether IGF2BP3 KO HSCs are able to give rise to normal blood production following the stress of transplantation.*

**Response:** Thank you for your suggestion. We have included new data from competitive transplantation assays, which suggests that KO of Igf2bp3 does not significantly affect normal blood production following the stress of transplantation (RL-Fig.19, also see Figs 6H-6K in the Revision).



**RL-Fig.19** Competitive transplantation assays. (A) Schematic outline of competitive repopulation assay.  $n = 7$  mice per group. (B) Flow cytometry analysis for different donor-derived cells (CD45<sup>+</sup>) in PB of recipient mice 4, 8, 12, and 16 weeks after BMT. (C and D) Percentage of donor-derived stem cell (C) and progenitor (D) compartments in the bone marrow of recipients 16 weeks after BMT.

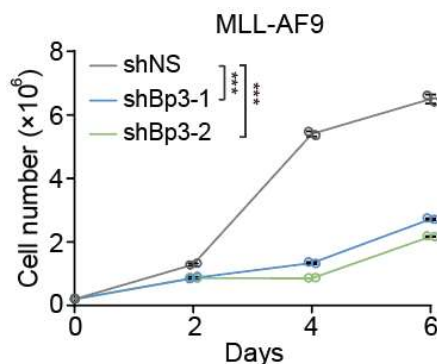
**Comment 12 from Reviewer #2:** *Given the impressive data shown with the shRNA experiments + SG-free diet in primary transplant, can the authors perform secondary transplantation with these cells into recipient mice with and without SG-free diet? This will determine if the anti-leukaemic phenotype has memory to the SG-free diet, or would relapsed patients, for example, have to be a SG-free diet long term to be disease free?*

**Response:** Thank you for your suggestion. It should be noted that our primary transplantation with shRNA followed by SG-free diet only provide proof-of-concept

results that targeting IGF2BP3 could be a promising therapeutic strategy in combination with SG-free diet. However, these results are preliminary. In addition, it is difficult to specifically knockdown IGF2BP3 in AML patients so far. Therefore, the proposed experiment is difficult to provide too much information for guiding clinical use of the combined therapy. It will be helpful to design IGF2BP3 targeting compounds and test whether it would have synergistic effect with SG-free diet. We have added related discussion in the revised manuscript.

**Minor Comment 1 from Reviewer #2:** *Related to the data presented in Figure 4, is there a proliferation defect seen in the murine MLL-AF9 cells, similar to that of the KD cells in THP-1s?*

**Response:** Yes, Igf2bp3 knockdown resulted in a proliferation defect in MLL-AF9 transformed murine HSPCs (RL-Fig.20, also see Fig S4C in the Revision), similar to the effect in human AML cell lines.

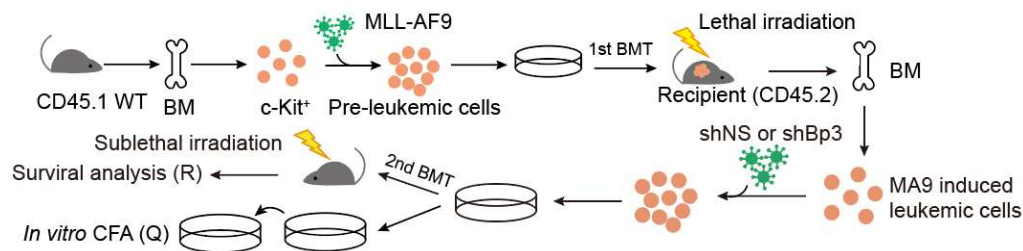


**RL-Fig.20** Knockdown of Igf2bp3 suppresses cell growth in MLL-AF9 transformed HSPCs.

**Minor Comment 2 from Reviewer #2:** *It is not clear where the cells for the secondary transplant are coming from. Can the authors include this in a schematic and consider moving these experiments further up the figure with the earlier knockdown experiments?*

**Response:** We have followed your suggestion to provide a schematic showing the origin of the cells for the secondary transplantation (RL-Fig.21, also see Fig 4P in the Revision). To maintain the logical flow of showing all the results of primary

transplantation before those of secondary transplantation, we prefer to keep the secondary transplantation results where they were (Figs 4P-4R).



**RL-Fig.21** Schematic showing the murine bone marrow transplantation assays.

### 3. Responses to the comments of Reviewer #3:

**General comments from Reviewer #3:** *The authors identify through CRISPR/Cas9 screen that IGF2BP3 is an important player in the persistence of AML and contributes to satisfying AML's high metabolic demand. By examining metabolic reprogramming through an epigenetic lens, the authors identify that IGF2BP3 recognizes m6A modifications on mRNAs encoding serine synthesis pathway enzymes and stabilizes these mRNAs, allowing for increased serine biosynthesis. The authors focus on the importance of serine biosynthesis, and the regulation of this serine synthesis pathway via m6A modifications to mRNAs encoding key enzymes (i.e. PHGDH, PSAT1) involved in this pathway. In this manuscript, the authors make the following claims:*

- (1) *Both extracellular serine and serine biosynthesis are necessary for AML cell growth.*
- (2) *IGF2BP3 and METTL14 are important in regulating serine metabolism, and loss of either sensitizes AML cell to serine deprivation-induced growth arrest.*
- (3) *IGF2BP3 regulates de novo serine biosynthesis, and that IGF2BP3 is utilized in AML cells to increase serine biosynthesis.*
- (4) *IGF2BP3 regulates serine biosynthesis by recognizing m6A modifications of mRNAs encoding PHGDH and PSAT1 and stabilizing these transcripts/increasing their expression, ultimately leading to increased serine production.*
- (5) *IGF2BP3 knockdown inhibits leukemogenesis in vivo.*
- (6) *IGF2BP3 is crucial for the maintenance of AML LSCs/LICs.*
- (7) *IGF2BP3 is dispensable for normal hematopoiesis.*

*(8) Knockdown of IGF2BP3 in combination with serine deprivation inhibits primary AML cell growth and improves survival of mice.*

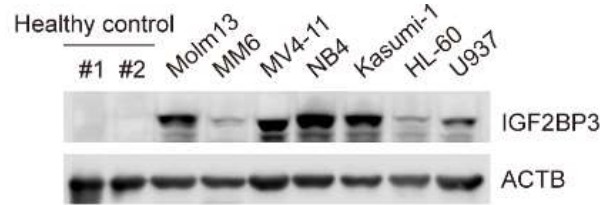
*This manuscript is well written and conveys important findings about a novel regulatory pathway for serine metabolism in AML. Strong points in the methodology include the use of an auxin-inducible degree system, the genetic knockout mouse model, the metabolic flux experiments, and direct characterization of IGFBP3 binding to its target mRNAs using RIP-seq. However, loss of IGFBP3 affects many other important pathways that are known to affect AML survival; therefore, it is unclear whether the effects on AML growth and survival are mainly driven by serine metabolism or other targets. In addition, the experiments are mostly performed in selected models that may not be representative of a general effect in AML.*

**Response:** We sincerely appreciate your positive appraisal of our manuscript and for the constructive comments/suggestions.

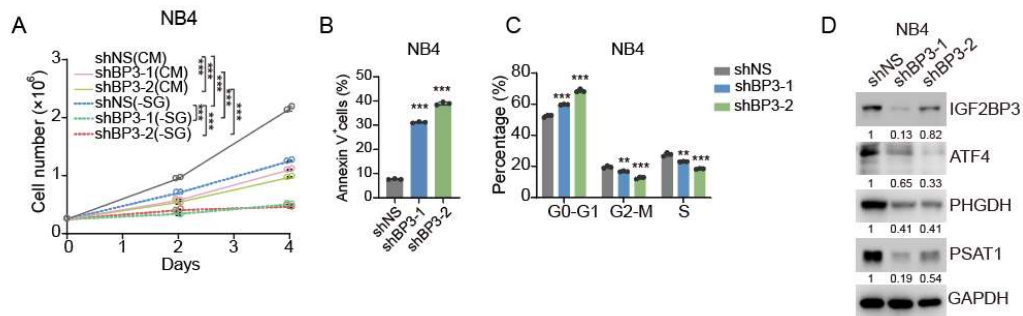
**Comment 1 from Reviewer #3:** *The findings are heavily based on MLL-rearranged models (human cell lines and mouse syngeneic), which have the highest expression of IGF2BP3 (Weng et al. Cancer Cell 2022). Whether loss of IGF2BP3 similarly affects AML growth and serine metabolism across most cases of AML, as is implied in the title, is unclear. Both findings should be validated in additional AML models representative of the genetic diversity of the disease before making a general claim. The experiments performed using primary AML samples may alleviate this concern, however they are lacking details regarding the genetic subtype, number of cases, etc.*

**Response:** Thank you for your suggestion. It should be noted that in our Cancer Cell 2022 paper you mentioned, we investigated IGF2BP2 but not IGF2BP3, and showed that IGF2BP2 has the highest expression in MLL-rearranged AML. This pattern was not observed for IGF2BP3, as our western blotting suggests that IGF2BP3 was highly expressed across AML cell lines representing different subtypes (RL-Fig.1, incorporated into the revision as Fig S1K). In this revision, we have added data from NB4 cell line (RL-Fig.2, also see Figs 1H, 3A, S3E and S3F in the revision). Moreover, FLT3-ITD/NPM1-mut and PML-RARa models have been added in our CFA assays

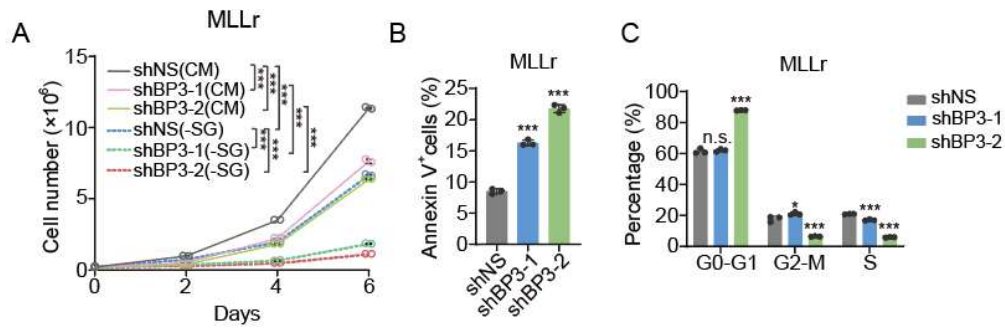
(RL-Fig.4, also see Figs 4E and 4F in the revision). Regarding to our primary AML samples, we initially used patient sample with FLT3-TKD, and now added another patient sample with MLL-AF9 in the revision (RL-Fig.3, also see Figs S3E, S3G, and S7C). The mutational status of the patient samples has been indicated in the corresponding figures.



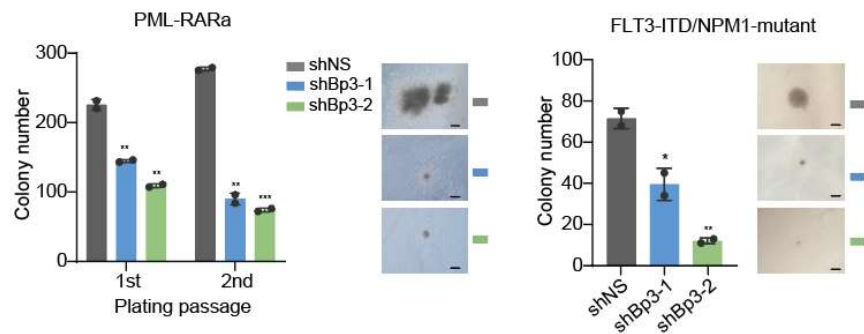
**RL-Fig.1** Western blotting of IGF2BP3 in CB MNCs from healthy donors as well as in cell lines.



**RL-Fig.2** Phenotypes of knocking down IGF2BP3 in NB4 cells. (A) Growth curves of control or IGF2BP3 KD NB4 cells in CM or -SG medium.. (B) Percentages of Annexin V<sup>+</sup> apoptotic cells in NB4 cells with or without *IGF2BP3* knockdown as detected by flow cytometry.. (C) Percentages of different cell cycle phases in NB4 cells with or without *IGF2BP3* knockdown as detected by flow cytometry. (D) Western blot showing expression change of ATF4, PHGDH and PSAT1 after *IGF2BP3* KD in NB4 cells. GAPDH was used as a loading control.



**RL-Fig.3** Phenotypes of knocking down IGF2BP3 in AML PDX cells with MLLr-rearrangement (MLLr). (A) Growth curves of AML PDX cells with MLLr upon *IGF2BP3* KD in CM or -SG medium. (B) Percentages of Annexin V<sup>+</sup> apoptotic cells in AML PDX cells upon *IGF2BP3* knockdown as detected by flow cytometry. (C) Percentages of different cell cycle phases in AML PDX cells upon *IGF2BP3* knockdown as detected by flow cytometry.

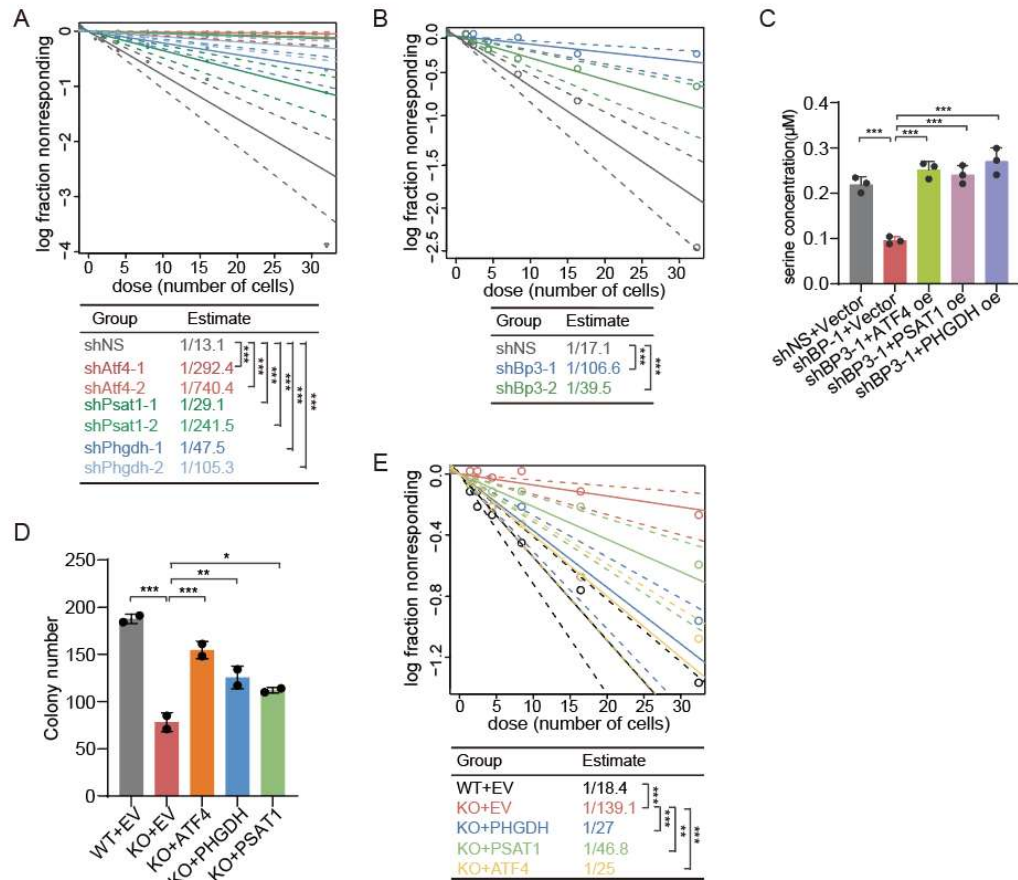


**RL-Fig.4** Knockdown of IGF2BP3 inhibits colony formation of mouse HSPCs immortalized by PML-RARa (left) or BM cells from FLT3-ITD/NPM1-mut mice (right).

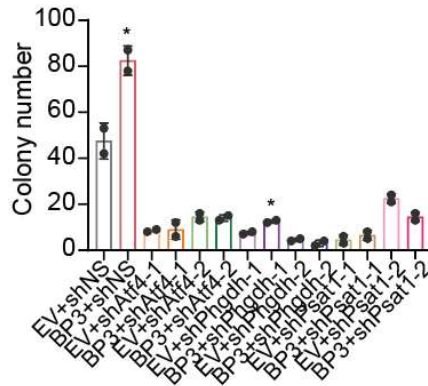
**Comment 2 from Reviewer #3:** Among the genes identified but not discussed by the authors, *BCL2* and *ATF4* are known to be involved in survival and the response to limiting amino acids levels, respectively. *MYC* and glutamine uptake were previously reported as *IGF2BP3* targets (36306790). *IGF2BP3* also enhances the stability of an apoptotic inhibitor (*RCC2*) (PMID: 35217832). These targets and others could potentially explain the effect seen on leukemogenesis. Therefore, the claim that augmentation of serine biosynthesis by *IGF2BP3* drives stemness is not completely proven; rather, *IGF2BP3* is important for leukemogenesis and *IGF2BP3* activity promotes serine metabolism in the MLL/AF9 model. I would have liked to see that

*overexpression of Igf2bp3 confers better engraftment and stemness than control MA9-transduced HSPCs, and that this effect is abrogated by perturbation of serine synthesis.*

**Response:** In Fig 5M, we showed that knockdown of SSP-related genes, including Psat1, Phgdh, and Atf4 (added in the revision), significantly reduced the stemness of MLL-AF9 transformed HSPCs, similar to the knockdown of Igf2bp3 (Fig 5L). More importantly, partial restoration of serine synthesis by ectopic expression of SSP genes (Fig 3M) partially rescued the loss of stemness of MLL-AF9 transformed HSPCs caused by Igf2bp3 KO (Figs 4O and 5N). These data (RL-Fig.22) suggest that IGF2BP3 regulated serine synthesis is critical for the stemness of MLL-AF9 transformed HSPCs. Following your suggestion, we performed additional CFA assays by overexpressing IGF2BP3 followed by knocking down SSP genes. As expected, IGF2BP3 overexpression promoted colony-forming capability of MLL-AF9 transformed HSPCs, and this effect was abrogated by knockdown of SSP genes (RL-Fig.23). BTW, MYC and glutamine uptake were previously reported as IGF2BP2 targets, not targets of IGF2BP3 (PMID: 36306790).



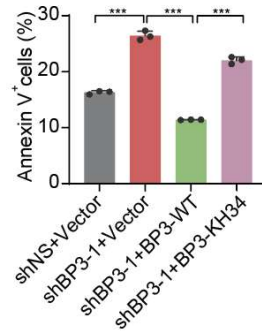
**RL-Fig.22** IGF2BP3 regulated SSP-related genes mediates AML stemness. (A, B) *In vitro* LDA. Logarithmic plots showing the percentage of nonresponding wells at different doses of mouse HSPCs co-transduced with *MA9* plus shRNA targeting *Igf2bp3* (A) or its target genes (B). (C) Serine level in Molm13 cells co-transduced with control or *IGF2BP3* shRNAs and ATF4/PHGDH/PSAT1 overexpression vectors. (D) HSPCs from WT or *Igf2bp3* KO mice were co-transduced with *MA9* and ATF4-, PHGDH-, or PSAT1-overexpressing viruses and seeded for CFA. (E) HSPCs collected from *Igf2bp3* WT and HO mice were co-transduced with *MA9* and target gene-overexpressing viruses and selected in methylcellulose medium with G418 and puromycin for 7 days before seeded for LDA assays.



**RL-Fig.23** IGF2BP3 overexpression promoted colony-forming capability of MLL-AF9 transformed HSPCs, and this effect was abrogated by knockdown of SSP genes.

**Comment 3 from Reviewer #3:** *It does not appear that the specificity of the shRNAs against IGF2BP3 was validated using rescue overexpression experiments. As a more general comment, it is unclear why some experiments in the murine MLL/AF9 model were performed with shRNA knockdown (i.e. Fig 5K,L, Fig 6F,G) when knock-out mouse models are available (genetically cleaner).*

**Response:** To avoid the off-target effect of shRNAs, we employed two shRNAs in all our experiments and observed similar effects, which is widely accepted in the research community. Moreover, we applied the auxin-inducible acute degradation model and the Igf2bp3 KO model, which also achieved similar results to those caused by shRNAs-mediated IGF2BP3 knockdown. To further address your concern, we also performed a rescue overexpression experiment, in which the IGF2BP3-sh1 (shBP3-1) shRNA was used because it targets the 3'UTR but not CDS of IGF2BP3 and thus will not lead to suppression of the ectopically expressed IGF2BP3. Knockdown of IGF2BP3 by shBP3-1 shRNA resulted in an increase of apoptosis in Molm13 cells, which could be completely rescued by ectopic expression of wildtype IGF2BP3 (BP3-WT), but not by that of KH3-4 mutated IGF2BP3 (BP3-KH34) that is deficient in binding m<sup>6</sup>A modification<sup>12</sup> (RL-Fig.24). These results not only confirm the specificity of the shRNA, but also suggest that the inhibitory effect of IGF2BP3 knockdown on AML cells is dependent on its role as an m<sup>6</sup>A reader.

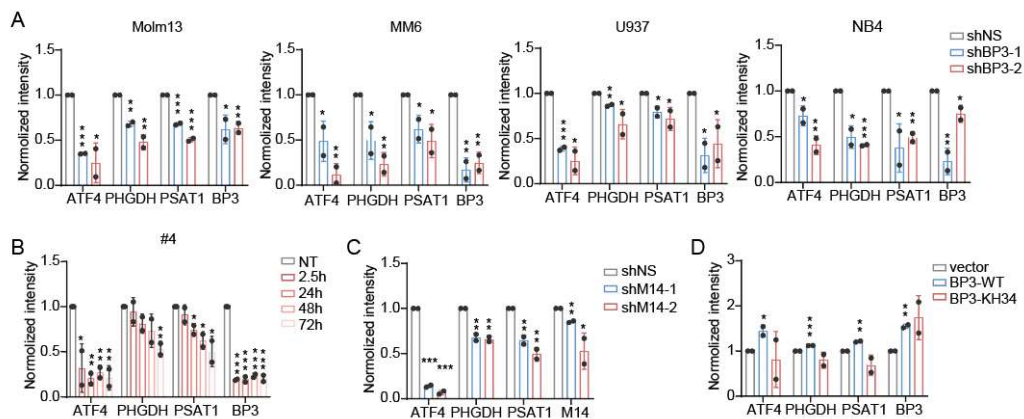


**RL-Fig.24** Rescue experiment to validate the specificity of IGF2BP3 shRNA (shBP3-1).

In the original Fig 5K (labeled as Fig 5L in the revision), we used shRNAs to knock down Igf2bp3 and evaluate the effect on the stemness of MLL-AF9 transformed murine HSPCs. This result, together with that in the original Fig 5J (labeled as Fig 5K in the revision) where the KO cells were used, confirmed that depletion of Igf2bp3 expression resulted in dramatic decrease of AML stemness. In the original Figs 6F and 6G (labeled as Figs 7E and 7F in the revision), we used shRNA-mediated knockdown instead of genetic KO, because we would like to provide proof-of-concept data that inhibition of IGF2BP3 could further improve survival when combined with SG deprivation, considering that inhibition (e.g., by inhibitors) of a protein rather than KO may be more applicable and safer in future clinical setting.

**Comment 4 from Reviewer #3:** *The claims of altered PHGDH and PSAT protein levels (Fig 3A-D) should be backed by quantification and statistical analysis.*

**Response:** We have followed your suggestion to quantify the band intensity of the western blotting results (RL-Fig.25).



**RL-Fig.25** Statistical analysis of Western blotting showing changes of IGF2BP3 target genes upon manipulation of IGF2BP3 or METTL14.

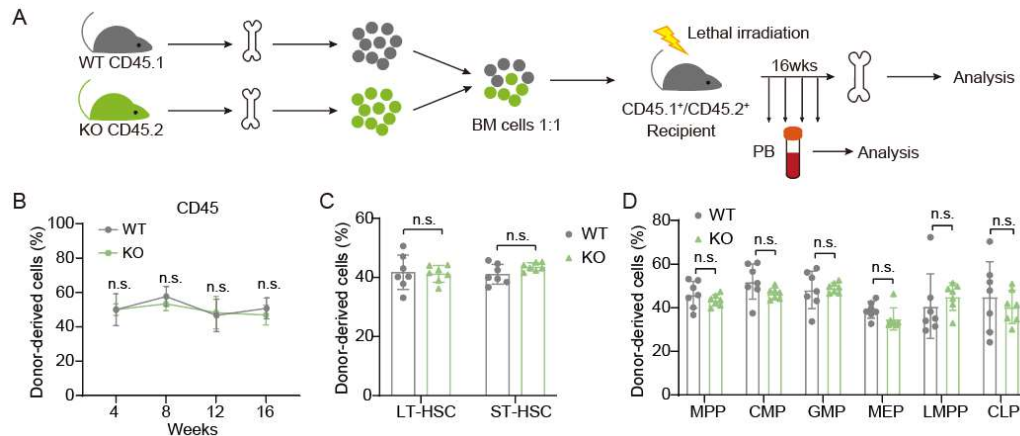
**Comment 5 from Reviewer #3:** line 310: "In line with the observed changes in the L-GMP population, KO of *Igf2bp3* 310 significantly decreased LSCs/LICs frequency (Fig 5J)". It is generally recognized that LSCs are functionally defined through *in vivo* assays. These should be performed, otherwise an alternate terminology should be used, i.e. "*in vitro* clonogenic potential".

**Response:** Thank you for your suggestion. The original Fig 5J (labeled as Fig 5K in the revision) displays the result of the *in vitro* limiting dilution assay, which is commonly used to evaluate the *in vitro* cell stemness by calculating frequency of LSCs/LICs-like cells<sup>13,14</sup>. Nevertheless, following your suggestion, we have now changed our expression from "LSCs/LICs frequency" to "*in vitro* stemness".

**Comment 6 from Reviewer #3:** line 322: "deletion of *Igf2bp3* does not affect HSC and multilineage development during normal hematopoiesis". Similar to 3), the gold standard definition of HSC is the ability to repopulate an irradiated recipient. Competitive bone marrow transplantation should be performed to claim that there is no impact on HSC numbers; otherwise this claim should be limited to "normal hematopoietic differentiation at steady state".

**Response:** Thank you for your suggestion. We have added new data related to the competitive bone marrow transplantation. Indeed, *Igf2bp3* KO does not affect HSC and

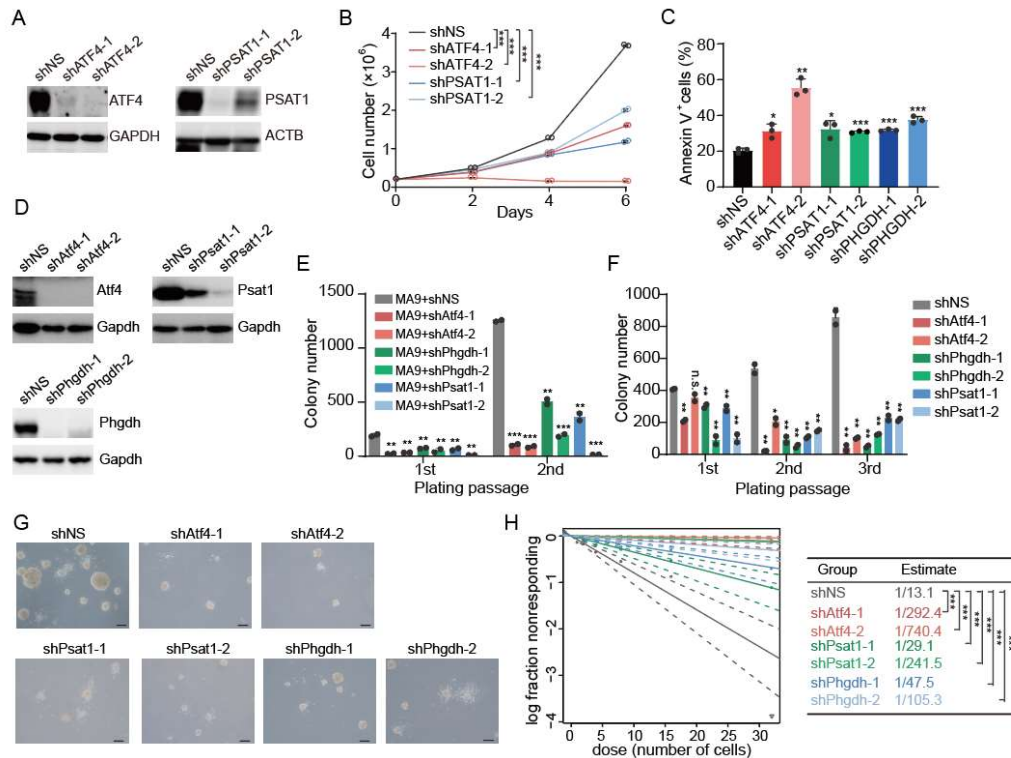
progenitor numbers compared to the wildtype cells upon irradiation-induced stress (RL-Fig.19, also see Figs 6H-6K in the revision). These data together with results shown in Figs 6A-6G suggest that deletion of *Igf2bp3* does not affect HSC and multi-lineage development during normal hematopoiesis.



**RL-Fig.19** Competitive transplantation assays. (A) Schematic outline of competitive repopulation assay.  $n = 7$  mice per group. (B) Flow cytometry analysis for different donor-derived cells (CD45<sup>+</sup>) in PB of recipient mice 4, 8, 12, and 16 weeks after BMT. (C and D) Percentage of donor-derived stem cell (C) and progenitor (D) compartments in the bone marrow of recipients 16 weeks after BMT.

**Comment 7 from Reviewer #3:** *The serine synthesis pathway has other key enzymes that are involved. Is it possible that decreased expression/activity of PHGDH could be inhibiting cell growth through a serine-independent pathway? To strengthen their argument, the authors should perform similar knockdown experiments with PSAT1 and PSPH to conclude that the SSP is equally crucial to AML survival/growth as extracellular serine.*

**Response:** Thank you for your suggestion. The results of PSAT1 knockdown were shown in parallel to PHGDH knockdown and ATF4 knockdown (RL-Fig.26, also see Figs S3I-3K, S4H, S4I, S4L and 5M in the revision). Because the expression of PSPH was not affected by IGF2BP3 knockdown/degradation, it was not considered as a target of IGF2BP3 and thus was not included in this study.

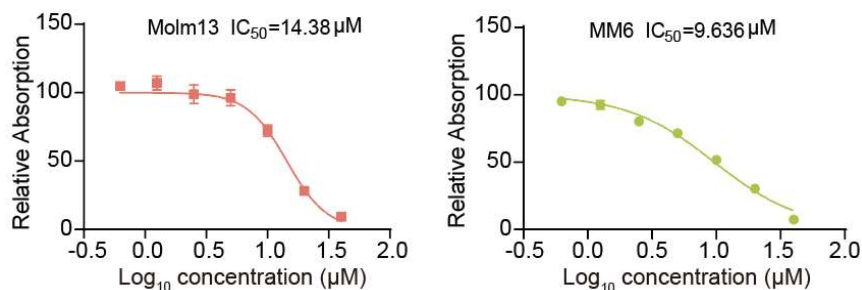


**RL-Fig.26** Knockdown of SSP related genes inhibits AML survival and stemness. (A) Western blot showing knockdown efficiency of shRNAs targeting *ATF4* or *PSAT1* in MM6 cells. (B) Growth curves of MM6 cells with or without KD of *ATF4* or *PSAT1*. (C) Percentages of Annexin V<sup>+</sup> apoptotic cells in MM6 cells with or without *ATF4*, *PSAT1*, or *PHGDH* knockdown as detected by flow cytometry. (D) Western blot showing knockdown efficiency of shRNAs targeting *Atf4*, *Phgdh*, or *Psat1* in MA9-transduced HSPCs. (E) Colony counts of mouse HSPCs immortalized by MA9 after KD of target genes. (F and G) CFA was performed using BM cells from MA9 leukemic mice transduced with shNS or shRNA targeting *Atf4*, *Phgdh*, or *Psat1*. Colony number was shown in (F), and the representative photos of colonies in CFA were shown in (G). (H) *In vitro* LDA. Logarithmic plots showing the percentage of nonresponding wells at different doses of mouse HSPCs co-transduced with *MA9* plus shRNA targeting *Igf2bp3* target genes.

**Comment 8 from Reviewer #3:** In Figure S1D, the authors show that the small molecule inhibitor of PHGDG reduces AML cell growth. They use a dose of 10  $\mu$ M of NCT-503. However, the authors do not contextualize the relevance of this specific dose.

*I would encourage the authors to add in a dose-response curve for NCT-503 for each cell line. It is necessary to rule out that the dose chosen does not induce cell death and/or off-target effects that could be inhibiting AML cell growth.*

**Response:** We have followed your suggestion to include dose-response curves for NCT-503 for Molm13 and MM6 cells (RL-Fig.27, also see S1D and S1E in the revision). The IC<sub>50</sub> values of NCT-503 after 2 days of treatment for each cell line were calculated and shown above the curves.



**RL-Fig.27** Dose-response curves for NCT-503 in Molm13 and MM6 cells after 2 days of treatment.

**Minor Comment 1 from Reviewer #3:** *In AML the RNA-binding protein YBX1, which binds to methylated (m<sup>6</sup>A) transcripts via IGF2BP-family of proteins (PMID: 33763698), impairs the translation of oncogenic transcripts (MYC, BCL2 and MCL1) and decreases growth and clonogenic potential of AML cells lines and patient samples (PMID: 34465866). A comment on this work needs to be incorporated in the discussion. Would YBX1 recapitulate IGF2BPs loss? Is YBX1, IGF2BP2 or other m<sup>6</sup>A binding proteins among the CRISPR screen hits?*

**Response:** Our CRISPR library was constructed in March 2021 before the publication of YBX1 being a cofactor of IGF2BP proteins became publicly available, therefore YBX1 was unfortunately not included in our CRISPR library. Nonetheless, we have included the related work in our Discussion section. We also have listed the dependency scores and SG dependent score increments for other m<sup>6</sup>A players. Among the m<sup>6</sup>A binding proteins, IGF2BP3 is the only one that has SG-dependent score increments of less than -0.1 between cell populations.

**Minor Comment 2 from Reviewer #3:** *The bibliography review related to the regulation of serine metabolism in AML is a bit superficial. For example, ATF4 (a hit in the screen) is known to regulate serine metabolism transcriptionally (33436370) - could this be a confounder? The authors do not refer to possible mechanisms hindered by PHGDH loss in limited serine/glycine availability (mTOR signaling downregulation, enhanced ROS levels, reduced protein synthesis).*

**Response:** Thank you for your suggestion. Considering the critical role of ATF4 in the regulation of serine metabolism, we have included it as a target of IGF2BP3 in the revision. We also followed your suggestion to discuss the potential inhibition of mTORC1 signaling and global protein synthesis upon IGF2BP3 KD-mediated SSP suppression.

**Minor Comment 3 from Reviewer #3:** *There is little mention of the possible role of IGF2BP3 in the translation of PHGDH and PSAT1 mRNAs, rather than stability. I believe that this data should be available from Weng et al. Cancer Cell 2022.*

**Response:** Thank you for your suggestion. Our Cancer Cell 2022 paper studied the role and mechanism of IGF2BP2, but not IGF2BP3, in AML. In this study, mRNA stability of ATF4, PHGDH, and PSAT1 was remarkably decreased upon IGF2BP3 KD, in line with and can explain the protein changes of these target genes. Nonetheless, we do not rule out the possibility of translation regulation and have included this in the Discussion section.

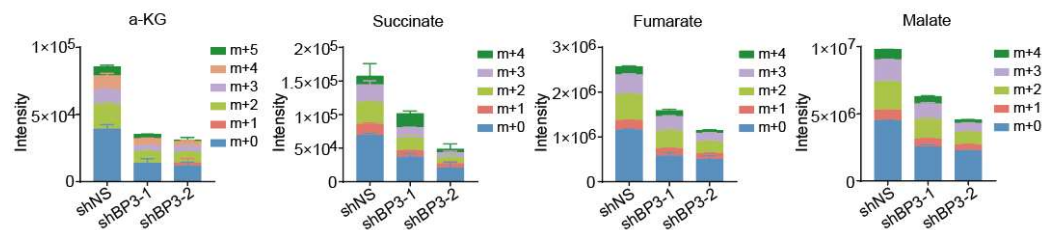
**Minor Comment 4 from Reviewer #3:** *For all growth curves, please add error bars and specify the sample size.*

**Response:** We have followed your suggestions to add error bars for all growth curves and specify sample size in the figure legends.

**Minor Comment 5 from Reviewer #3:** *The incorporation of C13-labeled glucose into the TCA cycle and connected metabolic pathways (i.e. highlighted in Fig 2C) should be*

reported in the supplementary. This would be especially helpful to understand if there is a specific effect on serine synthesis or other synthetic pathways are likewise affected.

**Response:** We have followed your suggestion to provide the incorporation of C13-labeled glucose into metabolites in the TCA cycle and connected metabolic pathways (RL-Fig.28, also see Fig S2A in the revision).



**RL-Fig.28** Changes of incorporation of C13-labeled glucose into metabolites in the TCA cycle and connected metabolic pathways upon IGF2BP3 KD.

**Minor Comment 6 from Reviewer #3:** Line 132. Remove “s” from Fig.

**Response:** Thank you for pointing this out. Correction has been made.

**Minor Comment 7 from Reviewer #3:** The authors do not reference Figure 1G in the text.

**Response:** Thank you for pointing this out. Correction has been made.

**Minor Comment 8 from Reviewer #3:** Line 143. Define acronym MOI.

**Response:** MOI is short for “multiplicity of infection”. This information has been added.

**Minor Comment 9 from Reviewer #3:** Line 160. Provide rationale for secondary IGF2BP3 inhibition strategy. Why was mRNA knockdown via sgRNA/shRNA not enough to demonstrate that IGF2BP3 inhibition suppressed AML survival/growth?

**Response:** The auxin-inducible degron version 2 (AID2) system can induce rapid depletion of the IGF2BP3 protein and thus can avoid potential secondary effects caused

by genetic knockdown/knockout. Therefore, it was utilized as an additional strategy to deplete IGF2BP3. We have included this rationale in the text.

**Minor Comment 10 from Reviewer #3:** *Line 182. No need to elaborate that citric acid cycle is also TCA cycle. Just write tricarboxylic acid cycle. No need for “TCA” abbreviation as TCA is only mentioned one more time in the text.*

**Response:** We have made changes as suggested.

**Minor Comment 11 from Reviewer #3:** *Line 184. Remove “considering the facts” and restructure this sentence. Does not flow.*

**Response:** The sentence has been rephrased to “Among these pathways, serine metabolism was noticed because it connects with other pathways such as the TCA cycle and are critical for the synthesis of purine, glutathione (GSH), and amino acids (Fig 2C), underscoring a potential central role of serine metabolism underlying the function of IGF2BP3.”

**Minor Comment 12 from Reviewer #3:** *Line 185. No need to provide acronym for alpha-ketoglutarate – never mentioned again in text.*

**Response:** Alpha-ketoglutarate has been deleted from the sentence due to restructuring.

**Minor Comment 13 from Reviewer #3:** *Line 191. Missing space before “Interestingly, ...”*

**Response:** Thank you for pointing this out. Correction has been made.

**Minor Comment 14 from Reviewer #3:** *Line 198. Is this the first report of m<sup>6</sup>A regulators controlling biosynthesis of an amino acid?*

**Response:** To our knowledge, this is the first report of m<sup>6</sup>A regulators controlling biosynthesis of serine.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

**Response:** Thank you both so much for your comments and suggestions.

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**SUMMARY:** We are very grateful to the Reviewers for their positive appraisal of our revised manuscript. We also sincerely appreciate the editor's decision to accept our manuscript for publication in principle.

**The following are our point-by-point responses to the remaining comments/suggestions from the Reviewer #3.** All the text in our revised manuscript that we have changed or added is marked in **RED**. The deleted text has been removed entirely from the manuscript.

**General comments from Reviewer #3:** *The authors have done a great job at improving the manuscript and addressing my concerns. Below are some remaining points that need to be addressed.*

**Response:** We sincerely appreciate your positive evaluation of our revised manuscript and the attention you dedicated to its review. We have addressed all the necessary corrections accordingly.

**Comment 1 from Reviewer #3:** *In addition to cell cycle arrest and apoptosis mediated by knockdown of IGF2BP3 in the MLL/AF9 models, could the authors comment if there is an effect on differentiation? The Wright-Giemsa stains from the BMT recipient mice (Fig 4C,N) exhibit morphological changes suggesting differentiation, but it is unclear if we are looking at sorted leukemic cells or total bone marrow (i.e. possibly including normal cells) - it would be very helpful to know how the cells shown in the panel "BM" were isolated. For this assay, ideally, sorted leukemic cells should be compared between conditions. Likewise, in the flow cytometric analysis in Fig S5C, it is unclear where the gating for CD45.1+ cells (i.e. transplanted AML) is done; so it is unclear if this is the immunophenotypic pattern of total bone marrow or leukemic cells.*

**Response:** In Figures 4C and 4N, BM cells were isolated from both the tibia and femur and loaded for cytospin preparation after lysing red blood cells. The purpose of BM cell staining is to assess the leukemia burden in recipient mice. Therefore, it is common practice in the research community to stain the entire population of nucleated cells rather than isolating leukemia cells for staining.

In Figure S5C, we did gate on CD45.2<sup>+</sup> cells (donor BM-derived AML cells) for analysis of L-GMPs, as indicated in the figure legend. The figure has also been labeled to reflect this information.

**Comment 2 from Reviewer #3:** *The rescue overexpression experiment (provided as figure RL-24 in the rebuttal) and the quantification of immunoblotting (RL-25) both address important technical concerns and should be included in the supplementary figures.*

**Response:** We have followed your suggestion to include these results into Figure S3. S3A-S3D, the quantification of immunoblotting; S3K, IGF2BP3 rescue experiment.

**Comment 3 from Reviewer #3:** *Could the authors add a commentary on the relationship between IGF2BP3 expression and cell growth/survival among AML cells? (specifically because they chose two cell lines (MOLM14 & NB4) which have relatively higher expression, and two cell lines (MM6 and U937) which have relatively lower expression for their assays).*

**Response:** We have shown in Figure S1K that expression of IGF2BP3 protein in AML cell lines is higher than normal cells. This can explain why KD of IGF2BP3 in all the tested AML cell lines caused growth inhibition.

**Minor Comment 1 from Reviewer #3:** *Line 36: add “the ” in front of mechanism*

**Response:** We have corrected it as suggested.

**Minor Comment 2 from Reviewer #3:** *Line 92: change “could ” to “can ”*

**Response:** We have corrected it as suggested.

**Minor Comment 3 from Reviewer #3:** *Line 107: add “a ” in front of synergistic effect*

**Response:** We have corrected it as suggested.

**Minor Comment 4 from Reviewer #3:** *Figure S1O: label that it is MOLM13*

**Response:** We have added the label as suggested.

**Minor Comment 5 from Reviewer #3:** *Line 191: “serine metabolism was [noted] because it…”*

**Response:** We have corrected it as suggested.

**Minor Comment 6 from Reviewer #3:** *Line 191: “Line 191-194: please rework this sentence. Very long and confusing.*

**Response:** We have rephrased this sentence into “Among these pathways, serine metabolism was noted for its connections with other key metabolic pathways (e.g., the TCA cycle) and its critical role in the synthesis of purine, glutathione (GSH), and amino acids<sup>10,11,51</sup> (Figs 2D and S2A), underscoring a potential central role of serine metabolism in mediating the function of IGF2BP3.”

**Minor Comment 7 from Reviewer #3:** *Line 225: “an enzyme [that] plays an…”*

**Response:** We have corrected it as suggested.

**Minor Comment 8 from Reviewer #3:** *Line 263: rescue instead of “rescued”*

**Response:** We have corrected it as suggested.

**Minor Comment 9 from Reviewer #3:** *Line 297: has instead of “have”*

**Response:** We have corrected it as suggested.

**Minor Comment 10 from Reviewer #3:** *Line 298: Was it ectopic expression of human genes (ATF4, PHGDH, PSAT1) or mouse (Atf4, Phgdh, and Psat1)?*

**Response:** We used human genes for the rescue experiments.

**Minor Comment 11 from Reviewer #3:** *Line 313: is instead of “was”*

**Response:** We have corrected it as suggested.

**Minor Comment 12 from Reviewer #3:** *Line 350-352: please correct this sentence.*

*Igf2pb3 is mouse, IGF2PB3 is human.*

**Response:** We have corrected it.

**Minor Comment 13 from Reviewer #3:** *Line 354: what is the proof for “stress hematopoiesis ” ? I would recommend adding stress of transplantation.*

**Response:** We have changed this sentence to “Collectively, these data demonstrate that deletion of Igf2bp3 does not affect HSC and multilineage development during steady-state hematopoiesis and under the stress of transplantation, highlighting Igf2bp3 as a safe therapeutic target.”

**Minor Comment 14 from Reviewer #3:** *Line 406: fix font discrepancy*

**Response:** We have corrected it.

**Minor Comment 15 from Reviewer #3:** *Line 408: replace “is ” with “the ”*

**Response:** We have corrected it as suggested.

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