Peer Review File

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Reviewer A

The authors showed that KMT2D facilitates lipid metabolism in prostate cancer through PPARγ and its downstream signals including ACC, ACLY, FASN. Lipid content in prostate cancer cell lines was reduced by knockdown of KMT2D probably due to inhibited PPARγ signaling pathway. These findings are interesting, but there are some caveats to be elucidated. Thus following contents should be considered to improve the manuscript.

Major comments

Comment 1: About line 199, Figure 1C merely shows the knockdown efficacy of siRNA. It is hard to know whether the KMT2D is related to lipid metabolism from this figure.

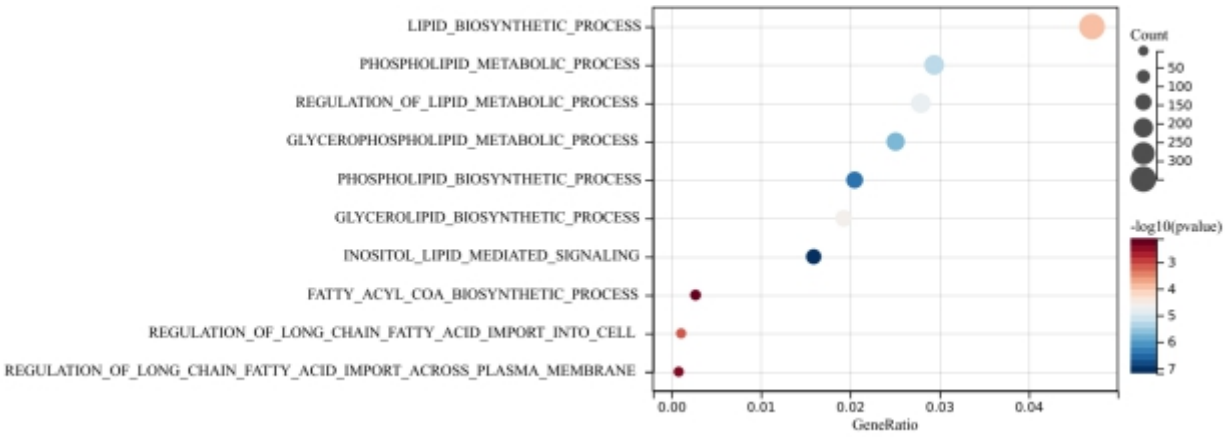
Reply 1: Thanks for your kindly reminder. We have revised the description in the "Results" section of the article. (see Page 10, line 203)

Comment 2: About line 209-212. Figure 2A, 2B compare the gene expression between prostate cancer and its normal counterpart tissue which is not about KDM2D upregulated sample and its normal counterpart. Thus, there is a severe logical leap to interpret that the results indicate the KMT2D high expression and its sequential change of lipid metabolism.

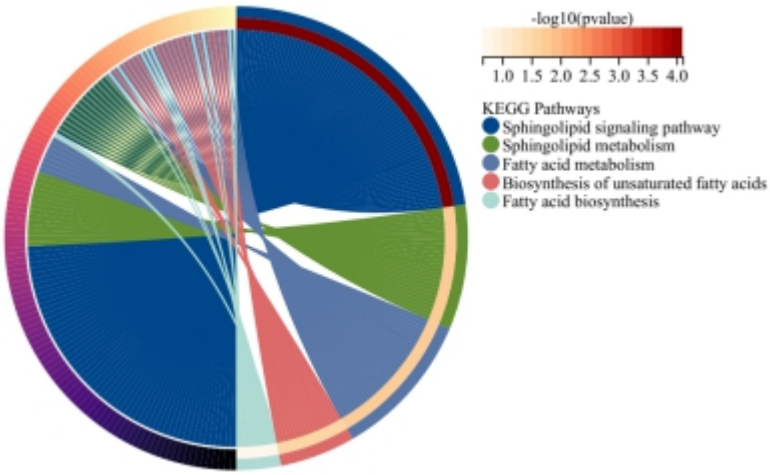
Reply 2: Thank you for pointing out this. After several attempts at grouping, we finally selected a new grouping for screening differentially expressed genes [two groups of tumor patients with high- and low-expression of KMT2D (cut of 50%), n=496], and functional enrichment analysis of DEGs (**Revised Figure 2A, GO analysis**; **Revised Figure 2A, KEGG analysis**) showed that KMT2D is associated with lipid metabolism and fatty acid metabolic pathways. (see Page 11, line 215)

Revised Figure 2A, B:

A



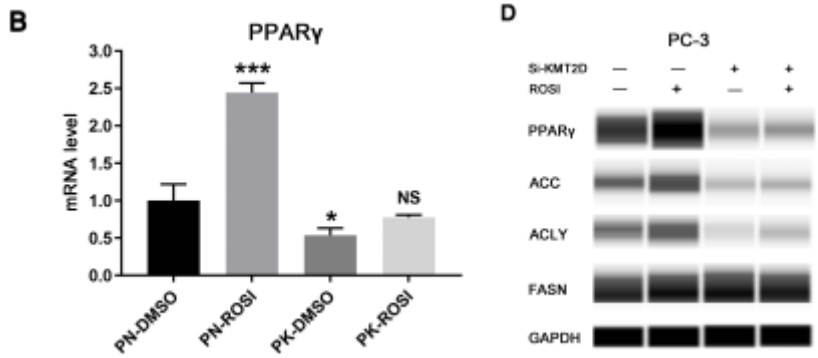
B



Comment 3: Please, show the PPARγ synthesis changes by ROSI

Reply 3: Thanks for the suggestion. As suggested, we have updated Figure 4 to add the PPARγ synthesis changes by ROSI. We found that compared with PN-DMSO cells, ROSI or DMSO treatment made the activities of PN-ROSI, PK-DMSO and PK-ROSI become 2.5 times, 0.5 times, 0.7 times (PN-DMSO expression level is set to 1). (see Page 12, line 244)

Revised Figure 4B, D:



Comment 4: How about the transcriptional change of PPARγ following the KMT2D knockdown? Line 234-236 indicates that KMT2D inhibits the lipid synthetic effect of ROSI by interrupting PPARγ. However. the authors evaluated nothing directly on KMT2D level or its activity in this content. Thus the presumable sentence contains caveats in the interpretation.

Reply 4: Thank you for pointing out this. Previous studies have found that the PPARγ itself is a target of the PPARγ-KMT2D axis [1]. As shown in "**Revised Figure 4B, D" and "Figure 5D, E",** PPARγ expression was downregulated when KMT2D was knocked down.

Reference

[1] Kim DH, Kim J, Kwon JS, Sandhu J, Tontonoz P, Lee SK, Lee S, Lee JW. Critical Roles of the Histone Methyltransferase MLL4/KMT2D in Murine Hepatic Steatosis Directed by ABL1 and PPARγ2. Cell Rep. 2016 Nov 1;17(6):1671-168.

Comment 5: In the DNA full down experiment, nothing about the relationship between KMT2D and PPARγ was elucidated. It is hard to say that KMT2D recruits PPARγ to its target genes.

Reply 5: Thanks for your kindly suggestion. It's important to elucidate the relationship between KMT2D and PPARγ, and actually in **Figure 5A**, we performed a Co-IP to detect the directly combation of KMT2D and PPARγ. As shown in **Figure 5B**, the PPRE motif of the fatty acid synthase (FASN) gene promoter region was predicted using Multiple EM for motif Elicitation (MEME), and DNA binding of KMT2D and PPARγ on the PPRE was confirmed by using the DNA pull-down assay, which was shown in **Figure 5C**.

Minor comments

Comment 1: In figure 1b expression levels of KMT2D and PPARγ look similar between cancer and normal cases. Furthermore, statistical analysis should be exhibited on this figure comparing two groups.

Reply 1: Thank you for pointing out this. We comprehensively reviewed the relevant experimental figures, and found that the labels for "Cancer" and "Normal" had been incorrectly placed. As the Human Protein Atlas database often only provides IHC images of the same patient, statistical analysis cannot be performed effectively. We refer to the manner of previous literature [1] to expand the expression of this data comparison. As shown in "**Revised Figure 1B" and Table S1,** we selected several representative images and summarized information on the intensity of staining for all images. In these tissues, the levels of the expression of KMT2D and PPARγ proteins were higher in tumor tissues than in normal tissues.

Revised Figure 1B:

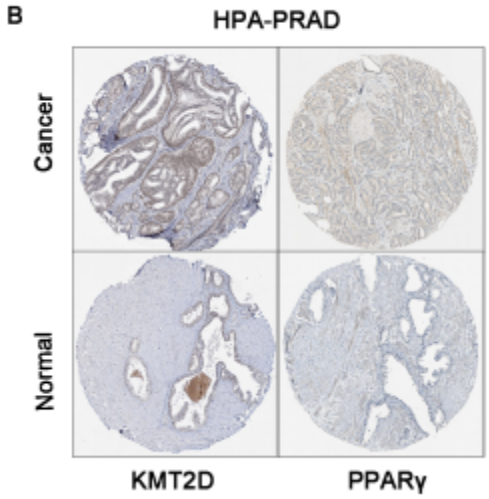


Table S1:

Immunohistochemical staining results of KMT2D and PPARγ proteins obtained by Human Protein Atlas in normal prostate tissues and prostate cancer tissues

Protein	Normal	Cancer (cases)				Antibody
		High	Medium	Low	Not detected	

KMT2D	Low	-	9 (90%)	1 (10%)	-	HPA035977
PPARγ	Not detected	-	-	7 (63.6%)	4 (36.4%)	CAB004282

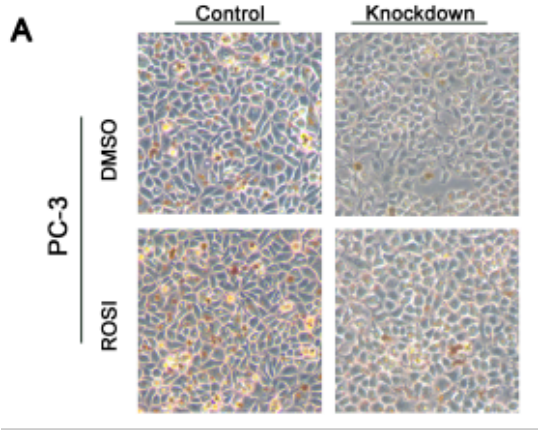
Referenc

[1] Xu M, Xu J, Zhu D, Su R, Zhuang B, Xu R, Li L, Chen S, Ling Y. Expression and prognostic roles of PRDXs gene family in hepatocellular carcinoma. J Transl Med. 2021 Mar 26;19(1):126. doi: 10.1186/s12967-021-02792-8.

Comment 2: Figure 4A does not show a significant increase of lipid droplets following ROSI treatment. More appropriate figures are required

Reply 2: Thank you for pointing out this. We comprehensively reviewed the relevant experimental figures, and re-replaced the **Figure 4A.**

Revised Figure 4A:



Reviewer B

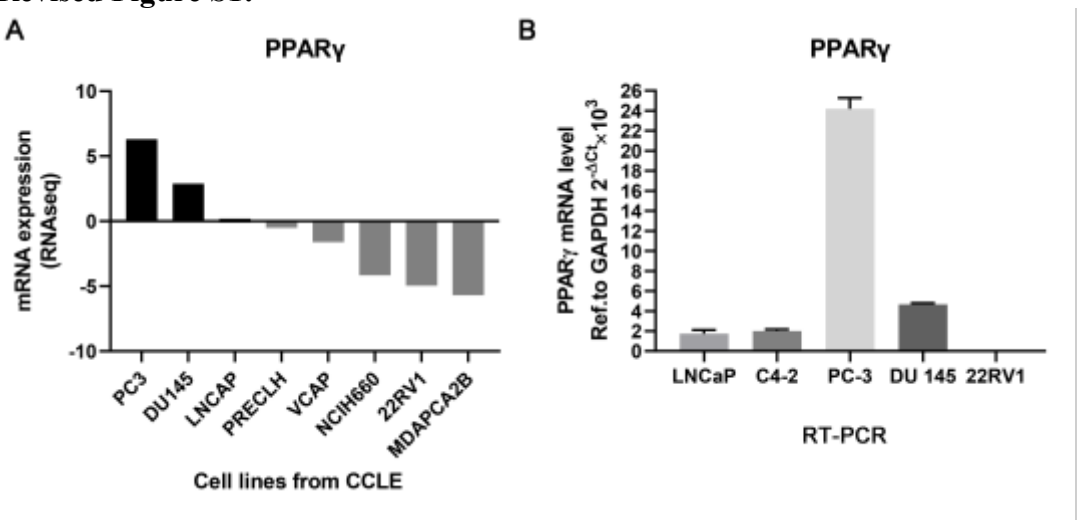
In this manuscript, the objective of this manuscript is to demonstrate that KMT2D was found to be directly involved in the regulation of PPARγ lipid metabolism, which affected PCa cell growth and proliferation. Mechanistically, KMT2D promoted oncogenic and abnormal lipid metabolism in PCa cell lines by forming a complex with PPARγ.On the whole, the authors reasonably carry on the experimental plan and the reported data. However, several issues need to be addressed:

Main points:

Comment 1: Can the authors explain the reason that PC3,DU-145,and LNCap have been chosen for this study. In addition, I noticed the two cell lines:PC-3 and DU-145 have been carried out for all of the experiments, but LNCap has been used only in Fig.5D. I prefer to show the relative data in LNCap and 22Rv1 cell lines in the study.

Reply 1: Thank you for pointing out this. Our study focused on KMT2D and PPARγ. Before selecting the cells, we first analyzed the transcript levels of PPARγ and KMT2D in PCa cell lines on the CCLE database, and the results are shown in Figure S1, which demonstrated that PPARγ was mainly expressed in PC3 and DU145. And we performed RT-PCR for PC3, DU145, LNCAP, C4-2, and 22RV1 cell lines (Figure S1B). We found that the transcript levels of PPARγ were similar to CCLE database. Especially, the PPARγ was almost not expressed in 22RV1 cell line. Although PPARγ is expressed at low levels in LNCAP and C4-2 cell lines, we still decided to abandon these two cell lines as this would more effectively bypass the effect of AR on the KMT2D- PPARγ axis. As described in our article, we would prefer to find a treatment independent of AR.

Revised Figure S1:

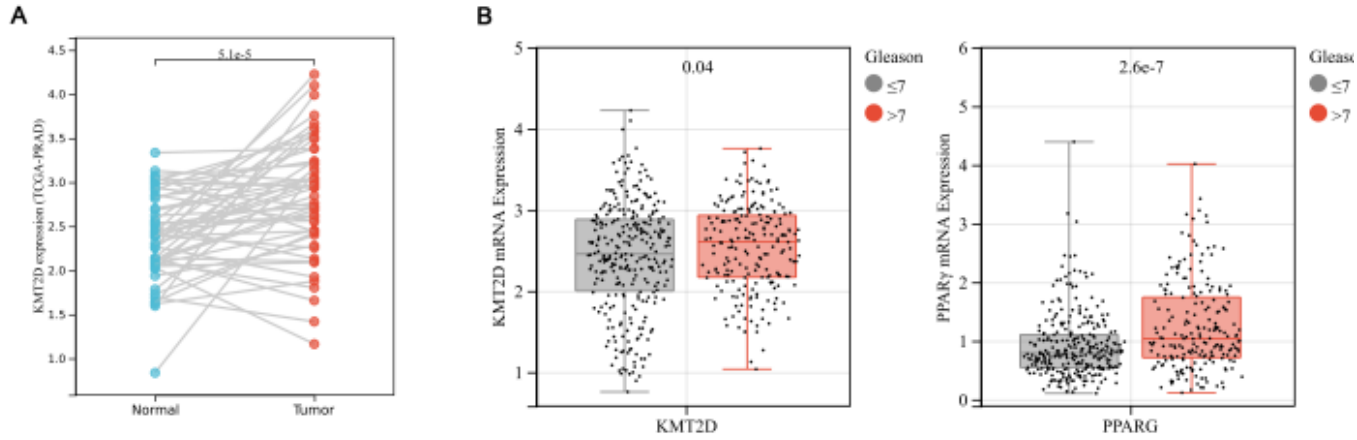


Comment 2: In Fig.1A, the authors can provide the data of KMT2D expression in other databases. In Fig1.B, how is the correlation between KMT2D and PPARγ with the tumor-grade of prostate cancer in the database of in PRAD. In Fig.1D, show the data of ORO staining in LNCap and 22Rv1 cell lines. In Fig.1E, the entire panel should be improved in terms of staining and resolution. scale bar missed in the pictures.

Reply 2: Thank you for pointing out this. As in **Figure S2A**, we have provided the data of KMT2D expression in TCGA databases. The data in **Figure 1B** were obtained from the HPA

database, which mainly presents immunohistochemistry of human tumors and para-cancerous tissue sections, but the number of patients is so limited that statistical analysis could not be performed. We have also performed further in-depth analyses (as described in "Reviewer A"). However, the point you made is meaningful. Then I analyzed the TCGA database and found that the expression of KMT2D and PPARγ increased significantly with the increase of Gleason score (**Figure S2B**). For the reasons stated in "Reply 1", our study did not explore the LNCAP and 22RV1 cell lines. In **Figure 1E**, we comprehensively reviewed the relevant experimental figures, and improved resolution (300dpi), and increased scale.

Figure S2:



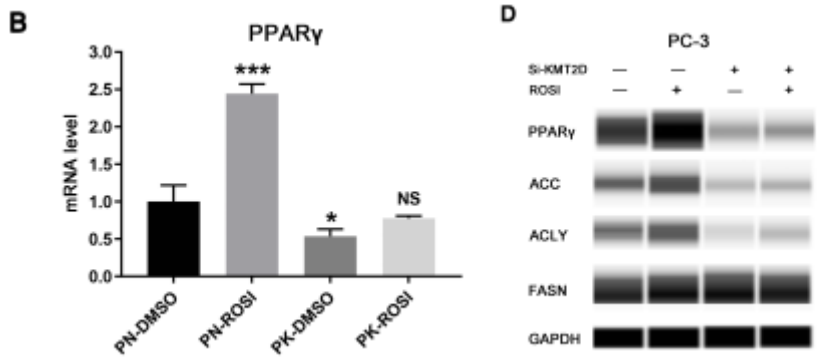
Comment 3: In Fig.3A, show the data of ACC, ACLY, and FASN in LNCap and 22Rv1 cell lines.

Reply 3: Thank you for pointing out this. For reasons as stated above, we would prefer to find a treatment independent of AR, our study did not explore the LNCAP cell lines. And 22RV1 cell line did not express PPARγ, which was not suitable for our study.

Comment 4: In Fig.4A, the authors only show the data in PC3.Could you show the representative pictures and statistic data in DU-145? In Fig.4B, could the authors show the PPARγ/ KMT2D expressions among the four groups by western blot. Furthermore, the evidences would be more solid if the authors can show some data by rescuing si-KMT2D by overexpression of PPARγ,which are similar to Fig.4A.

Reply 4: Thanks for the suggestion. As suggested, we have updated Figure 4 to add the PPARγ/ KMT2D expressions among the four groups by RT-PCR. We found that compared with PN-DMSO cells, ROSI or DMSO treatment made the activities of PN-ROSI, PK-DMSO and PK-ROSI become 2.5 times, 0.5 times, 0.7 times (PN-DMSO expression level is set to 1). Due to the direct and indirect effects of the COVID-19 pandemic, my laboratory is unable to carry out high-level experiments normally, and we cannot complete the "statistic data in DU-145 "and "rescuing si-KMT2D by overexpression of PPARγ" suggested by you. However, PC3 has the highest expression of PPARγ, which is more conducive to the exploration of the relationship between KMT2D and PPARγ. Therefore, we believe that the study on PC3 is sufficient to prove the question we raised. I hope you can understand our difficulty and this decision.

Revised Figure 4B, D:



Comment 5: The weakest aspect of the paper is that no evidence show the changes of PPARγ expression and lipid metabolism-related genes in xenograft tumor models of KMT2D KO in vivo. The authors should set up the experiment in vivo to compare the PPARγ expression and PPARγ target genes. The author groups have set up the xenograft models of KMT2D KO in vivo in Shidong Lv et.al Oncogene 2018 Mar;37(10):1354-1368. They also can use the previous samples for the analysis above.

Reply 5: Thanks for the suggestion. The survival rate of KMT2D KO xenograft tumor model is low. In previous studies, those animals have been killed and all samples have been used up. Our research team are building a new animal model, which will take a long time. We can't finish the experiment you suggested. I hope you can understand our difficulty and this decision.

Minor points:

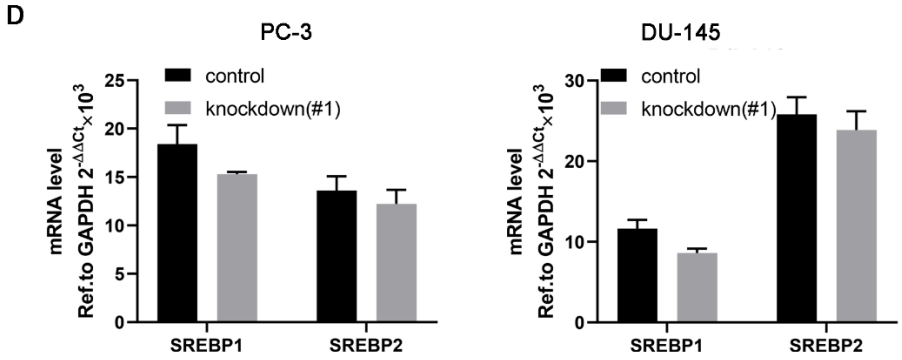
Comment 1: The authors should consistently use the format of ref. to $GAPDH^{2-\Delta ct}$ to show the value in Y axis for the mRNA level as Fig 3A and B. So, change the format of Fig.1C, Fig.3D, and Fig.4B.

Reply 1: Thanks for the suggestion. We have modified **Figure 3D** according to your suggestion. For the other two figures, especially the revised **Figure 4B, C**, in order to better compare the treatment group and the control group and identify their multiples of change, we still chose to keep the format. (The manuscript describes "Further analysis of the transcriptional expression level of PPARγ, we found that compared with PN-DMSO cells, ROSI or DMSO treatment made the activities of PN-ROSI, PK-DMSO and PK-ROSI become 2.5 times, 0.5 times, 0.7 times (PN-DMSO expression level is set to 1)"). Both of these formats have been effectively evaluated [1] and applied in a large number of literatures.

Reference

[1] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.

Revised Figure 3D:



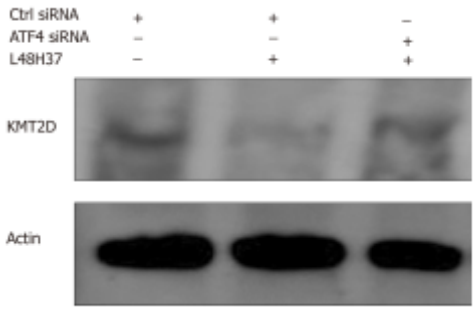
Comment 2: The sizes of text should be consistent in Figures.
Reply 2: Thank you for pointing out this. We checked all the figures and modified them as you suggested.

Comment 3: In Fig.3C, missed the label of knockdown (#1).
Reply 3: Thank you for pointing out this. We have corrected the figures as you suggested.

Comment 4: In Fig.5A and B, improve the quality of western blot, especially KMT2D. Make the right label: IB/IP

Reply 4: Thank you for pointing out this. KMT2D is a 592kDa protein, which is very difficult to be presented by western blot. In many literatures, their pictures are similar to our results [1]. We reviewed all of our relevant experimental pictures, we believed that the present picture was the best one. And we have modified the label according to your suggestion.

KMT2D (Proteintech; #27266-1-AP) [1]



Reference

[1] Li SS, Jiang WL, Xiao WQ, et al. KMT2D deficiency enhances the anti-cancer activity of L48H37 in pancreatic ductal adenocarcinoma. World J Gastrointest Oncol. 2019;11(8):599-621.