nature portfolio

Peer Review File

Epigenome-wide impact of MAT2A sustains the androgen-indifferent state and confers synthetic vulnerability in ERG fusion-positive prostate cancer

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
By performing meta-analysis of patient data, the authors discovered that the histone modification regulator MAT2A is upregulated in CRPC and NEPC. Transcriptome and cistrome analysis showed that MAT2A affects cell cycle and EMT gene expression and regulates chromatin accessibility, especially in the AR targeting sites. They further showed that MAT2A interacts with ERG and regulates prostate cancer cell growth. Finally, they tested the anti-cancer effects of MAT2A inhibitors in ERG fusion positive prostate cancer cells in vitro and in vivo.

Overall, the findings are interesting and suggest that MAT2A could be a prostate cancer relevance gene. However, several issues remain to be addressed for further improvement.

It is hard to see the details of the staining in Figure 1G. High magnification images are needed for both H&E and IHC. Similarly, high magnification images are needed for Figure 1L. Importantly, it seems the staining positivity, especially Ki67 staining in ERG/MAT2A group in Figure 1L does not match well with the quantitative data.

Based on the data shown in Figures 1A-G, it is so preliminary to conclude that MAT2A sustains stemness and tumor initiating capability of ERG fusion-positive tumor cells. More evidence is needed to substantiate the role of MAT2A in regulating prostate cancer stemness. Also, where is the evidence showing MAT2A is involved in tumor initiation? It is hard to understand the specific role of ERG fusion positive tumors without comparing its effect in fusion negative cells.

Based on the results shown Figure 3, the authors indicate that interfering with MAT2A might redirect AR to canonical target genes, reversing the aberrant activation of non-canonical AR targets in CRPC cells. However, as shown in Figures 1H-L, MAT2A alone was unable to promote sphere growth and tumor growth in mice. So, what is the role of ERG in the role of MAT2A in the redirection of AR binding in the genome as shown in VCaP cells?

By performing the high resolution mass spectrometry, the authors discovered that depletion of MAT2A largely decreased H3K4me2 in VCaP cells? This finding is very interesting, but how to understand the specific effects of MAT2A on H3Kme2? Any reasonable explanation here? Also, can this phenomenon be observed in other cell lines and is this effect is ERG dependent?

The authors indicate that MAT2A and ERG interact directly. In addition to the microscale thermophoresis (MST) assay using a cell-free condition, can this result be validated in independent assays? Also, proteasome inhibitor PS341 treatment studies suggest that MAT2A regulates ERG protein stability. Can this result be confirmed using different proteasome inhibitors and does MAT2A affect ERG ubiquitination? Importantly, what is the mechanism by which MAT2A regulates ERG protein degradation given that MAT2A is not an ubiquitin E3 ligase? Any thoughts and evidence?

The anti-tumor effects of the MTA2A inhibitors are intriguing. Is this ERG fusion cell specific? Also, how were the ERG, EZH2 and/AR target genes affected in response to the treatment of these inhibitors?

Reviewer #2:
Remarks to the Author:
In this study, Cacciatore et al focus on the role of MAT2A, the catalytic component of the SAM synthetase complex, as a promoter of androgen indifference in ERG-fusion prostate cancer. They report that MAT2A upregulation drives noncanonical AR activity and the development of neuroendocrine features. They used 2 inhibitors of MAT2A to demonstrate the therapeutic efficacy of

targeting MAT2A in ERG-fusion prostate cancers and downstream transcriptional consequences that may result in sensitization to AR and EZH2 inhibitors. Most of the mechanistic studies were done in a single cell line and mechanistic details on how MAT2A regulates AR indifferent state and key experiments to strengthen the translational rationale of MAT2A inhibition are lacking.

Specific comments:

• One of the main aspects of the manuscript is the influence of MAT2A on AR indifferent state and the acquisition of NE features. However, MAT2A is not significantly overexpressed in NEPC as compared to CRPC (Figure 1A). Is MAT2A only required for NE-initiation and not maintenance?
• What is correlation of MAT2A with other molecular features in patients beyond ERG (eg., AR expression, NE markers, stemness, etc). Since ERG mRNA expression is lost in TMPRSS2-ERG fusion positive, AR-negative CRPC/NEPC tumors, these implications should be discussed.
• The majority of the mechanistic studies were performed in a single cell line, VCaP. The authors should validate MAT2A related finding in other models including NEPC models as well as ERG fusion negative models. The H660 cell line harbors the TMPRSS2-ERG-fusion (but lacks AR and ERG expression). Since H660 is a small-cell AR indifferent NE line, the authors will be able to evaluate the influence of MAT2A on AR indifferent state. Additionally, the authors should use ERG fusion negative lines such as LNCaP to confirm the specificity of MAT2A influence of ERG- positive lines.
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• they report that NEPC genes are repressed in MAT2Akd VCaP cells - but VCAP is not a NEPC cell line. What was expression of classical NE genes in (eg INSM1, chromogranin, synaptophysin) at mRNA and protein level.
• Figure 2- Cumulative gene expression level of down-regulated genes in MAT2Akd VCaP cells in the clinical cohorts-- should include details on how was cumulative gene expression determined and what cutoffs used
• The authors demonstrate that H3K4me2 is specifically impacted by MAT2A silencing. MAT2A is a global regulator of methylation as it catalyzes the key methyl donor and therefore MAT2A silencing should impact a number of methylation patterns in the cell. The authors do not provide any explanation for perplexing induction of K9me2/3 and K14me2 upon MAT2A silencing. Subsequently, does the significant changes in K9me2/3 and K14me2 impact the oncogenic transcriptional program?
• LuCaP 35 spheroids/PDX —should confirm pre and post MAT2A, AR, EZH2, NE gene , MAT2A target gene expression at protein level.
• Figure 6F shows that there is only a moderate overlap between the MAT2A pharmacological inhibition and genetic silencing which raises concerns about the specificity of these inhibitors. The authors should address the specificity of this drug and also perform a MAT2A rescue experiment to validate on-target effects. Also, does pharmacological inhibition of MAT2A have the same effects at the epigenomic landscape?
• The authors have used MAT2A inhibition in combination with ADT and EZH2 inhibition. The authors argue that MAT2A inhibition counteracts ERG/EZH2 functional cooperation. The reviewer wonders whether there is a feed-forward loop between ERG/EZH2 and MAT2A that could be investigated. Does MAT2A inhibition specifically block the non-canonical function of EZH2? And is it necessary to combine it with a EZH2i in the clinic? Using a clinically relevant EHZ2i such as tazemetostat or valemetostat currently in clinical trials to validate their findings would strengthen the study.
• MAT2A is ubiquitously expressed in a number of tissues at high levels. The authors should comment on druggability of MAT2A and the specificity of the drug.

Reviewer #3:
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.

Reviewer #4:
Remarks to the Author:
This manuscript discussed the role of MAT2A as a driver of the androgen-independent state in ERG fusion CRPC. The results are relevant to the field. The manuscript is well written although there are some aspects that require further clarification. In particular, the chromatin analysis needs additional information. The results are represented mainly by piecharts and the signal needs to be shown by genome viewer and genome-wide heatmaps. In this manuscript, the authors describe unexpected results associating loss of H3K4me2 with accessible chromatin and with genes transcriptionally repressed, when H3K4me2 is a classic mark associated with chromatin transcriptionally active. Therefore, the results need to be further explained. Here are several points that need clarification:

1. The ATAC-seq and H3K4me2 characteristics for both control and MAT2A depleted cells should be summarized in a table showing number of reads sequenced, number of peaks, FRIP, and distribution in promoters, intergenic, exons, and introns.

2. The difference in ATAC-seq signal at AR locus shown in Figure 3G is not very convincing. The signal seems noisy, and it is not showing differential peaks, just a general subtle increase (for MAT2A depleted) that doesn't seems to correspond to enhancers or promoters. How is H3K4me2 at that region in control and MAT2A depleted?. It will be helpful to show both ATAC and H3K4me2 at AR locus. (The font should be also increased for the scale to be seen).

3. The reference to Figure 4C (line 246) for the lack of change in H3K27me3 is not correct.

4. The genome-wide distribution of H3K4me2 in MAT2A depleted and control VCaP cells shown in Figure 4D is not reflecting the typical distribution of the mark, which should also mark promoters (H3K4me2 marks promoters and enhancers). How many peaks are represented in that heatmap?, Are all the TSSs represented in rows?. The number of rows doesn't seem to be enough to cover all the TSSs (particularly obvious in the sh1 cells). Is that signal really centered at TSS?. This figure needs additional clarification.

5. In addition to the piecharts, It will be good to show examples of H3K4me2 peaks that are gained and peaks that are lost on the genome browser. Please, show examples of lost at enhancers and maintained peaks at promoters.

6. It Is surprising that both expression repressed and activated genes had a similar distribution of negative H3K4me2. H3K4me2 mark is associated with chromatin transcriptionally active. Again, I will suggest showing some examples by genome browser showing both the H3K4me2 signal and the RNAseq signal at representative examples.

7. Interestingly, the H3K4me1 signal is not reduced after MAT2A depletion. H3K4me1 is marking enhancers of chromatin transcriptionally active and overlaps with H3K4me2 at enhancers. It would be interesting to perform H3K4me1 and H3K4me3 to evaluate potential changes of these marks at enhancers and promoters respectively, in control and depleted cells. The fact that the signal is not significantly reduced, by mass spectrometry, doesn't necessarily mean that the signal cannot be redistributed. These marks (H3K4me1 and H3K4me3) should also be affected by MAT2A depletion and strongly overlap with H3K4me2 at enhancers and promoters respectively.

8. The authors mention that genes associated with H3K4me2 loss (for MAT2A depleted) are associated with pro-tumorigenetic and proliferative functions (line 262). This mark is reduced at intronic and

intergenic regions. The authors should explain how the gene assessment was done. Was It done based on distance to the lost peak?

9. Very importantly, the overlap between a reduced H3K4me2 and open chromatin (line 267) both at repressed and activated genes, is also very atypical. In general, accessible chromatin overlaps with H3K4me2 mark and is associated with expression. The authors should show examples, on genome browser, of reduced H3K4me2 at open chromatin for representative genes expressed or not expressed. And should show by a heatmap the overlap of H3K4me2 and ATAC-seq signal genome-wide.

10. Since the authors hypothesize that MAT2A could serve as a scaffold promoting ERG interactions with EZH2, I wonder how the authors envision the association between the activation of H3K4me2 and the binding of EZH2 (involved in placing the H3K27me3). H3K4me2 and H3K27me3 tend to be anti-correlated, except for bivalent promoters and enhancers. To assess this relationship, it would be relevant to also study H3K27me3 in MAT2A depleted and control cells in VCaP cells.

11. The authors show the expression of ERG and MAT2A expression by IHC in LuCaP 35 which is used to show the efficiency of the chemical inhibitors. It will be good to show an example of MAT2A expression by IHC in a model without TMPRSS2:ERG fusion, as a control to evaluate the relevancy of MAT2A expression in cases with ERG fusion.

Point-by-point response to the reviewer's comments

R=REVIEWER
A=ANSWER

Reviewer #1, expertise in prostate cancer epigenetics (Remarks to the Author):

By performing meta-analysis of patient data, the authors discovered that the histone modification regulator MAT2A is upregulated in CRPC and NEPC. Transcriptome and cistrome analysis showed that MAT2A affects cell cycle and EMT gene expression and regulates chromatin accessibility, especially in the AR targeting sites. They further showed that MAT2A interacts with ERG and regulates prostate cancer cell growth. Finally, they tested the anti-cancer effects of MAT2A inhibitors in ERG fusion positive prostate cancer cells in vitro and in vivo.
Overall, the findings are interesting and suggest that MAT2A could be a prostate cancer relevance gene. However, several issues remain to be addressed for further improvement.

R: It is hard to see the details of the staining in Figure 1G. High magnification images are needed for both H&E and IHC. Similarly, high magnification images are needed for Figure 1L. Importantly, it seems the staining positivity, especially Ki67 staining in ERG/MAT2A group in Figure 1L does not match well with the quantitative data.

A: We have improved the images relative to the histology of *in vivo* xenografts figure 1H and 1N (previous 1G and 1L). The new images are presented at a higher magnification and better resolution. The quantification scores shown in Figure 1N are correct (based on Aperio tool). We have included representative images to better show the increase in Ki67 staining only in RWPE-1 ERG+MAT2A xenografts, compared to RWPE-1 CTRL cell-derived xenografts.

R: Based on the data shown in Figures 1A-G, it is so preliminary to conclude that MAT2A sustains stemness and tumor initiating capability of ERG fusion-positive tumor cells. More evidence is needed to substantiate the role of MAT2A in regulating prostate cancer stemness. Also, where is the evidence showing MAT2A is involved in tumor initiation? It is hard to understand the specific role of ERG fusion positive tumors without comparing its effect in fusion negative cells.

A: We have amended the manuscript and explained more clearly our findings. Our data support that ERG and MAT2A act synergistically to promote tumor initiation and progression. Here, we highlight the role of MAT2A in the context of ERG fusion-positive tumors and investigate the mechanisms by which MAT2A cooperates with ERG in this context. We do not exclude that MAT2A might have a role in ERG fusion-negative tumors, but this would be mechanistically distinct and independent of ERG.

Our conclusion on the impact on stemness and tumor initiation is based on the data on tumor-sphere formation and tumor growth in VCaP cells. The tumor-sphere assay gives a functional assessment of the stem-like cell population in terms of their number, phenotypic characteristics, and self-renewal capability, and is a valid test of the cancer stem cell compartment. Tumor-sphere formation correlates with stem cell marker expression and tumor-initiating capability in mice. In vivo tumorigenesis assay consistently shows reduced tumor growth associated with the reduced presence of stem-like tumor cells in the in vitro assay.

In addition, we have gain-of-function studies in normal epithelial RWPE-1 cells, which we believe address even more directly the contribution of MAT2A and the cooperation with ERG. We have generated RWPE-1 cells with stable expression of MAT2A, ERG, and both ERG and MAT2A (Figure

1I). These normal prostate epithelial cells do not form tumors *in vivo* and are a suitable model to test early tumorigenic events (tumor-initiating capability). The *in vitro* (tumor-spheres) and *in vivo* (xenografts) data demonstrate the need for the concomitant expression of MAT2A and ERG to induce tumor-sphere growth *in vitro* and tumor formation *in vivo*, consistent with a stem-like phenotype and a synergistic interaction between MAT2A and ERG (Figure 1I-N). Relevantly, pharmacological inhibition of MAT2A did not affect the growth of RWPE1 cells. We have added these data in Figure S7D.

To further support these conclusions and the relevance of the ERG-MAT2A cooperation for the stem cell phenotype, we have evaluated stem cell markers in RWPE-1 cells. In line with our hypothesis, RWPE-1 co-expressing ERG and MAT2A have high levels of SOX2 and NANOG, not found in the control, MAT2A, and ERG-expressing cells. The new data are in Figure 1K of the revised manuscript.

Additional data present in the manuscript support that ERG and MAT2A compared to MAT2A alone enhance stemness. We performed RNA-seq of RWPE-1 cells with stable expression of ERG, MAT2A and ERG/MAT2A. Functional annotation revealed that the most enriched hallmarks in combined ERG/MAT2A cells were epithelial to mesenchymal transition (EMT) and Myc targets (Figure 2L). The induction of EMT in normal prostate epithelial cells is consistent with the enhanced cell plasticity and enhanced tumor sphere formation observed in cells with combined ERG and MAT2A expression compared to the other groups (Figure 1J). To highlight this point, in the revised figures, we have added a graphic showing selected CSC markers significantly enhanced by ERG+MAT2A ($p<0.05$) in stable cell lines (Figure 2M).

R: Based on the results shown Figure 3, the authors indicate that interfering with MAT2A might redirect AR to canonical target genes, reversing the aberrant activation of non-canonical AR targets in CRPC cells. However, as shown in Figures 1H-L, MAT2A alone was unable to promote sphere growth and tumor growth in mice. So, what is the role of ERG in the role of MAT2A in the redirection of AR binding in the genome as shown in VCaP cells?

A: The RWPE-1 model is not suitable for the impact on AR pathway. These cells are AR negative. The results in Figure 3 are relative to the VCaP model that is AR and ERG fusion-positive. Inhibition of MAT2A has a strong effect on AR pathway in these cells, supporting the notion of a synergism between ERG and MAT2A. This synergism is supported further by experiments showing a strong convergence between the genes modulated by MAT2A and ERG (Figure S5K), that MAT2A kd reduces ERG level and ERG activation (Figure S5J), and that MAT2A directly controls AR enhancer and AR target gene set along with ERG (Figure 3G-H).

R: By performing the high resolution mass spectrometry, the authors discovered that depletion of MAT2A largely decreased H3K4me2 in VCaP cells? This finding is very interesting, but how to understand the specific effects of MAT2A on H3Kme2? Any reasonable explanation here? Also, can this phenomenon be observed in other cell lines and is this effect is ERG dependent?

A: We have added data in other cell lines such as the ERG/PTEN prostate-derived cells (EPG2). Pharmacological inhibition in EPG2 significantly reduced H3K4me2 supporting the strong effect of MAT2A in regulating H3K4 methylation pattern (Figure S8D).

R: The authors indicate that MAT2A and ERG interact directly. In addition to the microscale thermophoresis (MST) assay using a cell-free condition, can this result be validated in independent assays?

A: In addition to MST (Figure S5E), the interaction of MAT2A and ERG was shown by co-immunoprecipitation and western blotting in two cell models: VCaP (Figure S5C) and RWPE-1 (Figure S5D). In the revised manuscript, we also added data in the murine EPG2 cells (Figure S8C),

R: Also, proteasome inhibitor PS341 treatment studies suggest that MAT2A regulates ERG protein stability. Can this result be confirmed using different proteasome inhibitors and does MAT2A affect ERG ubiquitination?

A: The experiment with PS341 supports that MAT2A regulates ERG stability. We performed previous testing with an additional proteosomal inhibitor MG-132 which is a covalent and irreversible inhibitor of the proteasome. We experienced enhanced cell death and thus performed the experiment with PS341. PS341 is a potent and reversible inhibitor of the proteasome and the experiment shown is a representative of multiple experiments.

R: Importantly, what is the mechanism by which MAT2A regulates ERG protein degradation given that MAT2A is not an ubiquitin E3 ligase? Any thoughts and evidence?

Our data support that MAT2A enhances ERG methylation, and ERG methylation in turn increases ERG stability. To address the request of the reviewers and understand the mechanisms by which MAT2A promotes ERG stability, we performed additional experiments using transfected ERG and MAT2A. Specifically, in the revised manuscript we evaluated ERG stability in RWPE-1 cells. We performed co-transfection experiments with MAT2A and wild-type ERG, or MAT2A, and ERG-K362A with the mutated methylation site (Zoma et al., 2021). Wild-type ERG was stable in the presence of MAT2A, whereas ERG-K362A was not. Thus, by promoting the methylation of ERG, MAT2A prevents ERG proteasomal degradation. These new data are shown in the revised figures in Figure S5H.

R: The anti-tumor effects of the MAT2A inhibitors are intriguing. Is this ERG fusion cell specific? Also, how were the ERG, EZH2 and/AR target genes affected in response to the treatment of these inhibitors?

A: We show in Figure S6F that pharmacologic inhibition of MAT2A decreases ERG and EZH2 protein levels. Importantly, also the methylated form of ERG (mERG) which is the most active and oncogenic form, was reduced. This is consistent with the effect of MAT2A genetic ablation. Thus, in ERG-positive tumors, MAT2A inhibition blocks the oncogenic active form of ERG. This vulnerability can be relevant in precision medicine approaches for treating ERG-positive tumors with aggressive phenotypes. However, our data do not exclude the effect of MAT2A inhibitors in ERG-negative tumors, as suggested by studies in other tumor types. What is novel here is the crosstalk between ERG and MAT2A, and the vulnerability of ERG-positive tumor cells to MAT2A inhibition.

Reviewer #2, expertise prostate cancer AR indifferent and organoids (Remarks to the Author):

In this study, Cacciatore et al focus on the role of MAT2A, the catalytic component of the SAM synthetase complex, as a promoter of androgen indifference in ERG-fusion prostate cancer. They report that MAT2A upregulation drives noncanonical AR activity and the development of neuroendocrine features. They used 2 inhibitors of MAT2A to demonstrate the therapeutic efficacy of targeting MAT2A in ERG-fusion prostate cancers and downstream transcriptional consequences that may result in sensitization to AR and EZH2 inhibitors. Most of the mechanistic studies were done in a single cell line

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Specific comments:

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A: We apologize if our statement was not clear on this aspect. We did not intend to demonstrate that MAT2A mediates the development of canonical neuroendocrine (NE) tumors, but it is involved in the progression to AR-independent/indifferent CRPC (including those with NE features, more properly defined as CRPC-NE). The main point is that MAT2A cooperates with ERG to drive ERG-positive PC to an androgen-independent castration-resistant state. In this context, we see that PC cells (VCaP) also express some NE features (i.e., expression of NE markers or genes upregulated in NEPC), which suggests they have a mixed/transitional phenotype, also seen often in clinical samples of CRPC that express both AR and NE markers. We show that MAT2A promotes the expression of the NE genes, and MAT2A knockdown reduces it. The proposed role of MAT2A is consistent with the increased expression of MAT2A in CRPC and CRPC-NE (Figure 1A), in line with a path from hormone-sensitive PC to CRPC with and without NE features (i.e., CRPC-NE). This is reflected in the heterogeneity of CRPC with multiple subtypes (e.g., AR-positive, NE-positive, and both AR- and NE-negative CRPC) and often with mixed marker expression.

One of the novel points of this work is that MAT2A promotes the expression of NE genes in canonical CRPC (CRPC-AR) cell models like VCaP cells (a non-NE model). We show that inhibiting MAT2A affects the expression of gene sets also overexpressed in NEPC/CRPC-NE, implicating MAT2A activation in the acquisition of alternative AR-independent features and transition to more aggressive CRPC variants.

R: What is correlation of MAT2A with other molecular features in patients beyond ERG (e.g., AR expression, NE markers, stemness, etc.). Since ERG mRNA expression is lost in TMPRSS2-ERG fusion positive, AR-negative CRPC/NEPC tumors, these implications should be discussed.

A: We show that MAT2A is high in both CRPC and NEPC, sustaining our conclusion that MAT2A is associated with castration resistance and evolution toward aggressive tumor phenotypes. We show that in CRPCs high MAT2A levels are associated with ERG-positive tumors (Figure 1B). In the revised manuscript, we included AR expression analysis to show the relationship between AR, MAT2A, ERG, and NE marker gene expression. There is no correlation between AR and MAT2A. MAT2A is high also in AR-low tumors. We have added a new heatmap of AR and MAT2A and the level of MAT2A in CRPC patients with high and low AR levels. These new data are in Figures S1A and B. There is also no significant correlation between MAT2A and NE markers in CRPC.

R: The majority of the mechanistic studies were performed in a single cell line, VCaP. The authors should validate MAT2A related finding in other models including NEPC models as well as ERG fusion negative models. The H660 cell line harbors the TMPRSS2-ERG-fusion (but lacks AR and ERG expression). Since H660 is a small-cell AR indifferent NE line, the authors will be able to evaluate the

influence of MAT2A on AR indifferent state. Additionally, the authors should use ERG fusion negative lines such as LNCaP to confirm the specificity of MAT2A influence of ERG- positive lines.

A: In this work, we performed mechanistic experiments in multiple ERG-positive tumor models. A multiomics characterization was performed in VCaP cells, but we cross-validated our findings in additional models including mouse-derived organoids from the ERG/PTEN mice (with the human TMPRSS2:ERG gene fusion knock-in and PTEN knock-out) and in a murine cell line (EPG2) derived from the same model. We also performed experiments in engineered RWPE-1 cells with expression of MAT2A and ERG to dissect the contribution of each player independently and in combination.

In this manuscript, we focused on the role of MAT2A in ERG fusion-positive PC. We showed evidence of functional and physical interaction between ERG and MAT2A. Therefore, we found it reasonable to investigate this aspect and explore the implications of their relationship. For this reason, we focused on models of ERG fusion-positive PC. Our objective was to characterize the ERG and MAT2A interaction and its consequences, including the possibility of treating ERG fusion positive tumors by exploiting their dependency on MAT2A activity. Indeed, our data strongly support the notion that ERG and MAT2A cooperate to promote disease progression and treatment resistance. This dependency on MAT2A creates a specific vulnerability to MAT2A inhibition that can be exploited for treating ERG positive tumors.

We are also looking at MAT2A in ERG-negative prostate tumors testing different prostate cancer models representative of various CRPC subtypes. However, these data are beyond the scope of this present manuscript, as we focus here on the implications of the ERG-MAT2A functional interaction and on the vulnerability of ERG-positive tumors to MAT2A inhibition. MAT2A may also play a role and be a relevant therapeutic target in ERG-negative CRPC and other tumors. In the revised manuscript we have explained more clearly that ERG/MAT2A crosstalk is not an exclusive mechanism of MAT2A-mediated oncogenesis.

R: Normal RWPE cells– what was histology (images not clear, could be magnified) and expression of stem/NE markers of RWPE after stable expression MAT2A, ERG, or both. The authors report that MAT2A drives NE differentiation but this is not shown in this model or other models.

A: We have improved the images relative to the histology of in vivo xenografts Figures 1H and 1N (previous 1G and 1L). The new images are presented at a higher magnification and better resolution. We have included representative images to better show the increase in Ki67 staining only in RWPE-1 ERG+MAT2A xenografts, compared to RWPE-1 CTRL cell-derived xenografts.

To further support these conclusions and the relevance of the ERG-MAT2A cooperation for the stem cell phenotype, we have evaluated stem cell markers in RWPE-1 cells. In line with our hypothesis, RWPE-1 co-expressing ERG and MAT2A have high levels of SOX2 and NANOG, not found in the control, MAT2A, and ERG-expressing cells. The new data are in Figure 1K of the revised manuscript.

Additional data already present in the manuscript support that ERG and MAT2A compared to MAT2A alone enhance stemness. We performed RNA-seq of RWPE-1 cells with stable expression of ERG, MAT2A and ERG/MAT2A. Functional annotation revealed that the most enriched hallmarks in combined ERG/MAT2A cells were epithelial to mesenchymal transition (EMT) and Myc targets (Figure 2L). The induction of EMT in normal prostate epithelial cells is consistent with the enhanced cell plasticity and enhanced tumor sphere formation observed in cells with combined ERG and MAT2A expression compared to the other groups (Figure 1J). To highlight this point, in the revised figures, we have added a

graphic showing selected CSC markers significantly enhanced by ERG+MAT2A ($p<0.05$) in stable cell lines (Figure 2M). NE markers were affected in VCaP cells but not in RWPE-1. Beware RWPE-1 are normal prostate epithelial cells and thus also with ERG+MAT2A it is unlikely to push progression toward an NE phenotype. However, our data support induction of EMT and CSC, relevant events related to cell plasticity and progression.

R: they report that NEPC genes are repressed in MAT2Akd VCaP cells - but VCAP is not a NEPC cell line. What was expression of classical NE genes in (eg INSM1, chromogranin, synaptophysin) at mRNA and protein level?

A: We examined the changes in gene expression in VCaP with MAT2A knockdown by RNA-seq. We examined the expression of the NE gene set. This set of genes was extracted from Beltran et al and is considered a set of NE genes that distinguish CRPC from CRPC-NE. VCaP cells represent a general model of CRPC-AR and are characterized by AR gene amplification. However, we found that various NE marker genes were expressed in these cells and were repressed upon MAT2A knockdown. Expression of NE marker genes (albeit at low levels) in PC cell lines is not surprising or unusual, attesting to the intrinsic heterogeneity of this cell line as seen also in many CRPCs. Importantly, the expression of NE markers, like SYP1 and INSM1, was downregulated by MAT2A knockdown, indicating that MAT2A could drive their activation. These data are included in Fig. 2K. Thus, MAT2A inhibition affects a set of target genes relevant to NE tumors, supporting the role of MAT2A in promoting a transition toward an NE phenotype.

R: Figure 2- Cumulative gene expression level of down-regulated genes in MAT2Akd VCaP cells in the clinical cohorts-- should include details on how was cumulative gene expression determined and what cutoffs used.

A: For cumulative gene expression levels, we utilized the same cutoffs as those we used in RNA-seq analysis (p-value adjusted ≤ 0.05 and Log2FC ± 1). The cumulative gene expression levels were computed with gsva function with "ssgsea" mode from GSVA package. Briefly, GSVA uses a non-parametric technique to measure the relative enrichment of gene sets across samples. GSVA converts a p-gene by n-sample gene expression matrix into a g-gene set by n-sample pathway enrichment matrix. We have added this information in the methods section of the manuscript.

R: The authors demonstrate that H3K4me2 is specifically impacted by MAT2A silencing. MAT2A is a global regulator of methylation as it catalyzes the key methyl donor and therefore MAT2A silencing should impact a number of methylation patterns in the cell. The authors do not provide any explanation for perplexing induction of K9me2/3 and K14me2 upon MAT2A silencing. Subsequently, does the significant changes in K9me2/3 and K14me2 impact the oncogenic transcriptional program?

A: We used mass spectrometry to examine histone PTMs. This approach gives a broad map of lysine methylation in histone proteins. The data revealed a robust effect of MAT2A knockdown on H3K4me2. Accordingly, we focused on H3K4me2 performing ChIP-seq and then convergence analysis with RNA-seq and ATAC-seq data. We also confirmed by Western blot the change in H3K4me2 in two ERG-positive cell lines (human VCaP and murine EPG2 cells), in which H3K4me2 was also affected by pharmacological inhibitors of MAT2A (Figure S7B for VCaP and Figure S8D for EPG2).

We are aware that potentially other methylation sites could be influenced by depletion or overexpression of MAT2A (refs), but we are also aware that the impact of MAT2A on specific histone marks could be cell-type dependent. The most robust change (log2FC >2 fold) we detected by Mass Spectrometry, and confirmed by western blot, was the reduction of H3K4me2. The impact of MAT2A inhibition would depend on the specific histone mark abundance, its dynamic regulation, and the type and abundance of enzyme depositing and removing the mark. Thus, in these CRPC cell models, depletion of MAT2A influences mainly the level of H3K4me2, which is abundant, actively and dynamically regulated and required to maintain active transcription of genes. Changes in other histone marks like K9me2/3 and K14me2 are of minor entity compared to H3K4me2. Other histone marks affected by the loss of MAT2A activity could have an impact on the transcriptional program of ERG-positive prostate cancer. However, their role is beyond the scope of this work.

R: LuCaP 35 spheroids/PDX —should confirm pre and post MAT2A, AR, EZH2, NE gene, MAT2A target gene expression at protein level.

A: We evaluated by IHC the protein levels of different markers in the xenografts from LuCaP 35 treated *in vivo* with AG-270 (Figure 5E). As previously indicated in the manuscript, we observed a significant down-regulation of ERG upon treatment with the MAT2A inhibitor. In the revised manuscript we included the IHC analysis of EZH2, AR and Chromogranin A. As expected from our *in vitro* studies, EZH2 protein levels are significantly affected and down-regulated by MAT2A inhibition. Conversely, AR is not affected by the inhibition of MAT2A. Finally, we evaluated the protein levels of a known NE marker, Chromogranin A (CHGA). Importantly, this marker is expressed at very low levels in LuCaP35, consistent with a more indolent phenotype of this patient-derived xenograft. Regardless, treatment with AG-270 significantly decreased the protein levels of CHGA, indicating that inhibition of MAT2A impairs the castration-resistant and neuroendocrine-like state of ERG-positive prostate tumors. These data are included in Figure 5G-H.

R: Figure 6F shows that there is only a moderate overlap between the MAT2A pharmacological inhibition and genetic silencing which raises concerns about the specificity of these inhibitors. The authors should address the specificity of this drug and also perform a MAT2A rescue experiment to validate on-target effects. Also, does pharmacological inhibition of MAT2A have the same effects at the epigenomic landscape?

A: We did not expect to have a complete overlap between genetic depletion (which is profound and persistent) and pharmacological inhibition (which is applied for a short time and transiently). Despite this, we found a highly significant overlap between the genes affected by MAT2A knockdown and the two MAT2A inhibitors. This is a striking result, speaking to the on-target effects of the compounds and to the reliability of downstream effects of MAT2A inhibition in these models.

These data indeed support the specific impact of the inhibitors on MAT2A regulated pathways. We also observed concordance on the effects on the histone PTMs between pharmacological inhibitors and genetic ablation of MAT2A. Pharmacological inhibition of MAT2A specifically decreases H3K4me2 leaving unaltered other histone methylation marks as seen with MAT2A knockdown in VCaP and EPG2 cells (Figure S7B and S8D, respectively). MAT2A inhibitors also affected ERG, mERG, and EZH2 protein levels (Figure S6F and S8B).

R: The authors have used MAT2A inhibition in combination with ADT and EZH2 inhibition. The authors argue that MAT2A inhibition counteracts ERG/EZH2 functional cooperation. The reviewer wonders

whether there is a feed-forward loop between ERG/EZH2 and MAT2A that could be investigated. Does MAT2A inhibition specifically block the non-canonical function of EZH2? And is it necessary to combine it with a EZH2i in the clinic? Using a clinically relevant EHZ2i such as tazemetostat or valemetostat currently in clinical trials to validate their findings would strengthen the study.

A: Our data support a synergistic cooperation between ERG/EZH2 and MAT2A activities. Indeed, mass spectrometry analysis in MAT2A-depleted VCaP cells show that H3K27me3 is not affected by loss of MAT2A. Additionally, we found that both loss and inhibition of MAT2A decrease the protein levels of mERG, the active form of ERG, which we previously reported to be promoted by EZH2 non-canonical methylation pattern (Zoma et al., 2021). Taken together these data, we can speculate that MAT2A loss of function specifically acts on non-canonical functions of EZH2. The EZH2 inhibitor used (GSK-343) is efficient and the data consistent with our hypothesis that MAT2A impacts on the ERG/EZH2 transcriptional network. In future studies *in vivo* we will include the most clinically relevant EZH2i.

Also, we showed that knockdown of ERG reduces EZH2 and MAT2A. Thus, ERG induction of MAT2A and EZH2 creates a feed forward loop. Knockdown or inhibition of MAT2A disrupts this loop, reducing ERG and mERG levels, and reducing EZH2 level and activity. However, MAT2A depletion does not affect global H3K27me3, the canonical substrate of EZH2. But impact ERG methylation, which is a direct consequence of noncanonical activity of EZH2.

R: MAT2A is ubiquitously expressed in a number of tissues at high levels. The authors should comment on druggability of MAT2A and the specificity of the drug.

A: In this work we used two different drugs with high affinity for MAT2A, one of which (AG-270) is currently used in clinical trials for the treatment of tumors characterized by loss of MTAP, supporting the notion that this MAT2A inhibitor is safe and specific for its target. When we treated LuCaP 35 tumor-bearing mice systemically with AG-270 we did not observe signs of toxicity or body weight loss (Figure S6I). Notably, we achieved significant tumor growth at a low dose of the drug (10 mg/kg).

Additionally, *in vitro* cell viability assays did not reveal relevant effects at increasing concentrations of MAT2A inhibitors (up to 62.5µM) in normal RWPE-1 cells. These data are shown in Figure S7D.

Reviewer #3, Early Career Research who co-reviewed with one of the main reviewers (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.

Reviewer #4, expertise in prostate cancer epigenetics (Remarks to the Author):

This manuscript discussed the role of MAT2A as a driver of the androgen-independent state in ERG fusion CRPC. The results are relevant to the field. The manuscript is well written although there are some aspects that require further clarification. In particular, the chromatin analysis needs additional

information. The results are represented mainly by piecharts and the signal needs to be shown by genome viewer and genome-wide heatmaps. In this manuscript, the authors describe unexpected results associating loss of H3K4me2 with accessible chromatin and with genes transcriptionally repressed, when H3K4me2 is a classic mark associated with chromatin transcriptionally active. Therefore, the results need to be further explained. Here are several points that need clarification:

A: We thank the reviewer for the positive comments. H3K4me2 is associated with transcriptionally active chromatin. MAT2A inhibition reduces H3K4me2 and is associated with the repression of protumorigenic genes in VCaP cells. These results are not unexpected. The novelty of this finding is that the repressed genes are preferentially androgen-independent genes, and the effect is linked specifically to MAT2A and H3K4me2 function in these cells. Chromatin accessibility measured by ATAC-seq does not change significantly at sites overlapping the repressed genes (open/repressed). Indeed, loss of H3K4me2 can be compatible with accessible chromatin by ATAC-seq and transcriptional repression by alternative mechanisms (e.g., impaired recruitment of transcriptional activators or increased binding of repressive factors).

R: The ATAC-seq and H3K4me2 characteristics for both control and MAT2A depleted cells should be summarized in a table showing number of reads sequenced, number of peaks, FRIP, and distribution in promoters, intergenic, exons, and introns.

A: The requested table indicating the information concerning ATAC-seq and ChIP-seq analysis is now included in the files containing all the raw data.

R: The difference in ATAC-seq signal at AR locus shown in Figure 3G is not very convincing. The signal seems noisy, and it is not showing differential peaks, just a general subtle increase (for MAT2A depleted) that doesn't seems to correspond to enhancers or promoters. How is H3K4me2 at that region in control and MAT2A depleted? It will be helpful to show both ATAC and H3K4me2 at AR locus. (The font should be also increased for the scale to be seen).

A: In the revised manuscript, we increased the font of the IGV software images already present in the paper. To robustly assess the presence of significantly differentiated peaks is preferable to refer to differential expression analysis providing the entity of increment/decrease (Log_2FoldChange) and the significance (padj). The number of differentially expressed peaks on the AR region has been included in the raw data availability file, and is referred to Figure S3D. Specifically, we evaluated the peaks with a Log_2FoldChange ≥ 1.5 and a padj ≤ 0.05. We provided the visualization on IGV (Integrative Genomics Viewer) software (Figure 3G), providing qualitative information, to highlight the presence of differentially expressed peaks within and upstream of the AR gene, including the promoter regions (the distribution of these peaks is reported in Figure S3D). As an example, in Figure S3E we provided the zoom on enhancers in which the difference between VCaP_Ctrl and VCaP_sh1 peaks is clearly visible. To visualize VCaP_Ctrl and VCaP_sh1 signals the same scale was adopted.

R: The reference to Figure 4C (line 246) for the lack of change in H3K27me3 is not correct.

A: We corrected the manuscript in the revised version.

R: The genome-wide distribution of H3K4me2 in MAT2A depleted and control VCaP cells shown in Figure 4D is not reflecting the typical distribution of the mark, which should also mark promoters

(H3K4me2 marks promoters and enhancers). How many peaks are represented in that heatmap? Are all the TSSs represented in rows?. The number of rows doesn't seem to be enough to cover all the TSSs (particularly obvious in the sh1 cells). Is that signal really centered at TSS? This figure needs additional clarification.

A: The overall distribution of the H3K4me2 peaks in MAT2Akd and control VCaP cells is shown in the pie charts in Figure 4F (negative peaks) and in Figure S4D (positive peaks). Here, we can appreciate the different distribution of H3K4me2 peaks on the promoter region of the genes. On the other hand, the heatmaps represent all the significant peaks that were called for each condition. Only the peaks around the TSS (-1500, +1500) were considered for visualization in the heatmap. Consequently, the TSS regions that do not present peaks were not represented in the heatmap in Figure 4D. Specifically, VCaP Ctrl samples have 15018 peaks, while VCaP Sh1 have 2203 peaks. We have added this information in the legend of Figure 4D, which presents the referred heatmap.

R: In addition to the piecharts, it will be good to show examples of H3K4me2 peaks that are gained and peaks that are lost on the genome browser. Please, show examples of lost at enhancers and maintained peaks at promoters.

A: In the revised manuscript and figures, we provided examples of differentially distributed H3K4me2 peaks on genes with open ATAC-seq peaks and either enhanced or decreased RNA-seq expression (Figure S4M-N). Specifically, we provide examples of the opening of the chromatin (ATAC-seq) and loss of H3K4me2 peaks (ChIP-seq) on genes with either repressed mRNA expression (DIAPH3, H4C3, PRC1 and UBE2C) or activated mRNA expression (KLF5 and ADAMTS1).

R: It is surprising that both expression repressed and activated genes had a similar distribution of negative H3K4me2. H3K4me2 mark is associated with chromatin transcriptionally active. Again, I will suggest showing some examples by genome browser showing both the H3K4me2 signal and the RNAseq signal at representative examples.

A: In the revised manuscript and figures, we provided examples of differentially distributed H3K4me2 peaks on genes with open ATAC-seq peaks and either enhanced or decreased RNA-seq expression (Figure S4M-N). On the one hand, we provide examples of DIAPH3, H4C3, PCR1 and UBE2C, genes with known oncogenic properties. These genes are characterized by open ATAC-seq peaks, loss of H3K4me2 ChIP-seq peaks and decreased RNA-seq expression. On the other hand, we then provide the examples of KLF5 and ADAMTS1, which instead have anti-tumorigenic properties: they are characterized by opening of the chromatin (ATAC-seq), loss of H3K4me2 peaks (ChIP-seq) and increased expression (RNA-seq). These examples indicate how the loss of H3K4me2 peaks is not necessarily associated with decreased transcription, as the general asset of chromatin organization can influence the binding of transcriptional activators or repressors.

R: Interestingly, the H3K4me1 signal is not reduced after MAT2A depletion. H3K4me1 is marking enhancers of chromatin transcriptionally active and overlaps with H3K4me2 at enhancers. It would be interesting to perform H3K4me1 and H3K4me3 to evaluate potential changes of these marks at enhancers and promoters respectively, in control and depleted cells. The fact that the signal is not significantly reduced, by mass spectrometry, doesn't necessarily mean that the signal cannot be redistributed. These marks (H3K4me1 and H3K4me3) should also be affected by MAT2A depletion and strongly overlap with H3K4me2 at enhancers and promoters respectively.

A: We understand the point of the reviewer. All the histone marks are potentially redistributed upon loss of MAT2A. However, in this study, we focused on the evaluation of the most affected histone mark upon MAT2A depletion. H3K4me1 was not affected by loss of MAT2A, neither in mass spectrometry nor in western blot. In future studies, we can carry out a comprehensive analysis of all the histone mark changes including mapping of their redistribution. Mapping the redistribution is not trivial and would require the complete analysis of all the other histone markers to produce an output consistent and clear.

R: The authors mention that genes associated with H3K4me2 loss (for MAT2A depleted) are associated with pro-tumorigenic and proliferative functions (line 262). This mark is reduced at intronic and intergenic regions. The authors should explain how the gene assessment was done. Was It done based on distance to the lost peak?

A: Each peak was annotated with the nearest gene on the chromatin. Specifically, the annotatePeak function from ChipSeeker package was used (2). We have added this information in the methods section of the manuscript.

R: Very importantly, the overlap between a reduced H3K4me2 and open chromatin (line 267) both at repressed and activated genes, is also very atypical. In general, accessible chromatin overlaps with H3K4me2 mark and is associated with expression. The authors should show examples, on genome browser, of reduced H3K4me2 at open chromatin for representative genes expressed or not expressed. And should show by a heatmap the overlap of H3K4me2 and ATAC-seq signal genome-wide.

A: We understand the concern of the reviewer. However, while the H3K4me2 mark is generally associated with active gene expression, it can also be present in repressed genes. Importantly, the local histone post-translational modification status can influence the activation or repression of specific genes. In the revised manuscript, we added a circular plot representing the overlap between ATAC-seq, RNA-seq and ChIP-seq signals as well as a heatmap representing the integration of ATAC-seq and ChIP-seq signals (Figure S4K-L). Furthermore, we show examples of open chromatin, ChIP-seq negative and RNA-seq up-regulated genes (KLF5 and ADAMTS1), and examples of open chromatin, ChIP-seq negative and RNA-seq down-regulated genes (DIAPH3, H4C3, PRC1 and UBE2C). KLF5 and ADAMTS1 are genes associated with a normal-like pro-differentiating status and tumor-suppressive role. In contrast, DIAPH3, H4C3, PRC1 and UBE2 are associated with a pro-tumorigenic role. We added these data in Figure S4M-N. Taken together, these data support that H3K4me2 has a pro-tumorigenic and pro-proliferative role, associated with a non-canonical AR transcriptional program. Importantly, by modulating the genome-wide distribution of H3K4me2, MAT2A has a key role in the definition of the androgen-indifferent state of ERG-positive prostate tumors.

R: Since the authors hypothesize that MAT2A could serve as a scaffold promoting ERG interactions with EZH2, I wonder how the authors envision the association between the activation of H3K4me2 and the binding of EZH2 (involved in placing the H3K27me3). H3K4me2 and H3K27me3 tend to be anti-correlated, except for bivalent promoters and enhancers. To assess this relationship, it would be relevant to also study H3K27me3 in MAT2A depleted and control cells in VCaP cells.

A: Our main hypothesis is that MAT2A impacts on non-histone function of EZH2 and enhances the EZH2-induced ERG methylation. Consistently, we did not observe any significant changes in H3K27me3 protein levels in MAT2Akd cells, compared to controls. In the revised manuscript, we performed a ChIP experiment followed by qPCR on H3K27me3 and evaluated its occupancy on the promoter of NKX3-1.

Considering the canonical AR target gene NKX3-1 which is also a tumor suppressor gene, upon MAT2A ablation, we found molecular changes indicating a more permissive local environment supporting activation of this target (observed with RNA-seq).

- Increased chromatin opening in the body and enhancer region (Figure 3H).
- Increased AR occupancy by ChIP-PCR on the promoter of NKX3-1 gene in MAT2A-depleted cells compared to control VCaP cells (Figure 3I).
- ChIP-PCR of H3K27me3 reduced on the promoter of NKX-3-1 (Figure 4M).

R: The authors show the expression of ERG and MAT2A expression by IHC in LuCaP 35 which is used to show the efficiency of the chemical inhibitors. It will be good to show an example of MAT2A expression by IHC in a model without TMPRSS2:ERG fusion, as a control to evaluate the relevancy of MAT2A expression in cases with ERG fusion.

A: We have data from prostate cancer patients addressing this question. MAT2A is highly expressed in CRPC and prevalently associated with ERG-positive tumors (Figure 1B). There are also cases of ERG-negative CRPC and NEPC expressing MAT2A at similar levels.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have made a great effort in revising the manuscript by performing additional experiments and some of the data are much convincing now such as MAT2A and ERG regulating cancer stemness. However, a number of issues remain outstanding.

The images for Figures 1H and 1N are much better now in
resolution. While the authors indicated that the quantification scores in Figures are correct based on Aperio tool. However, the quantification data show a two fold differences at most while the differences in ki-67 staining appear much bigger than that. What is the definition for the positivity of ki-67 staining here? Can the authors use a different tool for quantification to see whether they could get the similar quantification data?

As indicated by the authors, RWPE-1 cells are AR negative. In this case, it appears that the role of MAT2A in prostate tumorigenesis observed in RWPE-1 model is different from those in VCaP model, which is AR positive. Therefore, it is a big concern that the mechanism in driving prostate tumorigenesis observed in Figure 1 and those defined in Figure 3 are distinct. How do the authors reconcile this?

The authors were able to show that pharmacological inhibition of MAT2A in other cells also significantly reduced H3K4me2, which supports their previous observation. However, they left issues such as specificity of MAT2A inhibition in regulating H3K4me2 and the ERG dependency unaddressed, not even a discussion.

Reviewer #2:
Remarks to the Author:
The authors have made substantial revisions to address the Reviewer comments and have overall strengthened the manuscript. While the data are interesting and point to a novel role of MAT2A in modulating plasticity in ERG positive prostate cancer, additional prostate cancer models were not added at revision. Much of the work has been done comparing a benign cell line RWPE and one AR driven cancer cell line VCaP that does not typically develop lineage plasticity. And only ERG positive models were tested with MAT2A inhibitors. In order to propose a novel precision medicine strategy for ERG positive prostate cancer, at the very least the authors should show effects of MAT2A inhibition in ERG negative prostate cancer.

Specific comments:

1. As neither NEPC nor adeno models that transdifferentiate to NEPC are tested, the authors should revise statements, potentially supporting MAT2A for ‘NE-initiation’ (i.e. NE-transdifferentiation from CRPC-Adeno) and not for the maintenance of NEPC phenotype upon terminal differentiation.

2. In Figure 1B and 1C, MAT2A expression levels are only slightly higher in ERG positive tumors and the scale on y-axis should be adjusted to 0-15; the authors should provide statistical analysis to support MAT2A levels being higher in ERG positive tumors. The authors mention but do not show that MAT2A expression is not correlated with expression of NE markers.

3. Validating the findings regarding MAT2A in ERG-fusion-positive NE line, H660, and ERG-fusion-negative lines such as LNCaP, 22Rv1 is essential to develop a specific strategy for targeting MAT2A in ERG-fusion positive prostate cancer.

4. New revised data is interesting that MAT2A+ERG over-expression led to an increase in stemness markers SOX2 and NANOG. The authors should review their RNA-Seq data from VCAP cells and confirm whether these markers (at the protein level) are downregulated upon MAT2A silencing. This is important because MAT2A may be primarily mediating stemness and not necessarily NEPC differentiation.

5. VCaP is not an NEPC cell line however, at the RNA-level may express low levels of INSM1, SYP, and other NE makers. Changes in these transcripts after MAT2A knockdown or overexpression should also be shown at the protein level.

6. The authors satisfactorily addressed the comment on Figure 2 cumulative gene expression.

7. LuCaP35 is an AR-driven model that does not develop NEPC, and therefore its not clear how reduction in CHGA levels upon AG-270 impacts NE-trans-differentiation. Would also test NE model such as LuCaP 49, 93, 145, 173

8. The authors should include a short paragraph on the druggability and clinical translation of MAT2A inhibitor therapy in the Discussion section.

Reviewer #3:
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.

Reviewer #4:
Remarks to the Author:
The authors have satisfactorily addressed most of my concerns.

Point by point response

R: Reviewer
A: Answer
Changes in the revised manuscript are indicated and marked in blue.

Reviewer #1 (Remarks to the Author):
The authors have made a great effort in revising the manuscript by performing additional experiments and some of the data are much convincing now such as MAT2A and ERG regulating cancer stemness. However, a number of issues remain outstanding.

R: The images for Figures 1H and 1N are much better now in resolution. While the authors indicated that the quantification scores in Figures are correct based on Aperio tool. However, the quantification data show a two fold differences at most while the differences in ki-67 staining appear much bigger than that. What is the definition for the positivity of ki-67 staining here? Can the authors use a different tool for quantification to see whether they could get the similar quantification data?

A: We appreciate the comments of the reviewer and provided a new quantification of the data (see Figure 1M). It was not necessary to implement additional approaches as the tools provided with the Aperio suite allow multiple options for accurate quantification of digitalized images. In the revised figure we present the percentage of positive cells in the xenografts and compare the data with the control group. We also have increased the number of sections and fields (≥12/section) evaluated for each tumor xenograft. The quantitative analysis of multiple fields show a difference (≥4 fold) in Ki67 staining in the ERG+/MAT2A+ group. In addition, we want to point out that, with the exception of the ERG+/MAT2A+ group, the other xenografts form mostly normal-like prostatic structures and a minimal number of altered foci in the case of MAT2A+ cell xenografts. Only the ERG+/MAT2A+ xenografts show tumor-like lesions. These data are included in Figure 1N.
R: As indicated by the authors, RWPE-1 cells are AR negative. In this case, it appears that the role of MAT2A in prostate tumorigenesis observed in RWPE-1 model is different from those in VCaP model, which is AR positive. Therefore, it is a big concern that the mechanism in driving prostate tumorigenesis observed in Figure 1 and those defined in Figure 3 are distinct. How do the authors reconcile this?

A: The Reviewer disputes using RWPE-1 cells (a common model of non-tumorigenic prostate epithelial cells) because these cells do not express AR, and therefore differ from VCaP cells. Indeed, the scope of this gain-of-function experiment was to assess the ability of ERG and MAT2A to cooperate in normal prostate epithelial cells and in the absence of endogenous expression of ERG, MAT2A, and AR. The experiment shows increased stemness and tumorigenesis with the co-expression of MAT2A and ERG, compared to each protein alone.
We do not see contradictions between the two models. VCaP and RWPE-1 cells give complementary information: both experiments show the interaction and cooperation between ERG and MAT2A. Furthermore, the RWPE-1 experiment shows that the cooperation between MAT2A and ERG does not involve AR. Our interpretation of these data is that the ERG-MAT2A axis does not depend on AR. Instead, it bypasses AR signaling and sets the stage for AR independence and increased tumorigenicity. This finding is also in line with the MAT2A expression pattern in prostate cancer patients: MAT2A is high in CRPC, and MAT2A expression is independent of AR expression, suggesting a role in AR-independent CRPC tumors (see Figure 1A and Supplementary Figure 1A-B in the manuscript).

R: The authors were able to show that pharmacological inhibition of MAT2A in other cells also significantly reduced H3K4me2, which supports their previous observation. However, they left issues such as specificity of MAT2A inhibition in regulating H3K4me2 and the ERG dependency unaddressed, not even a discussion.

A: We understand the reviewer's concern. However, the link between MAT2A and H3K4me2, revealed by our work, is well supported by the data and indicates a specific relationship. We show that MAT2A controls H3K4me2 in ERG-positive prostate cancer cells. Inhibiting MAT2A by genetic and pharmacological tools reduces H3K4me2 preferentially relative to other histone marks. We also show that both MAT2A upregulation and H3K4me2 accumulation are associated with AR independence and castration resistance. These findings are relevant and in line with previous data showing that the H3K4me2 marks AR-independent genes and is preferentially enriched in CRPC cells (ref #22). Furthermore, the increase in H3K4me2 is a general phenomenon in CRPCs and the relationship with MAT2A is not limited to ERG-positive models. Indeed, we have addressed this last point with additional data included in the revised manuscript (Figures 9). Specifically, we evaluated ERG-negative prostate cell lines LNCaP and LNCaP^{abl}, which have different levels of MAT2A expression. Relevantly, the H3K4me2 histone mark correlated with MAT2A expression and was higher in the castration-resistant LNCaP^{abl} cells. Also, 22RV1 cells with low MAT2A expression had a low level of H3K4me2. Furthermore, inhibiting MAT2A in LNCaP^{abl} cells reduced the H3K4me2 level, which is consistent with a direct impact of MAT2A on this histone modification. Finally, MAT2A^{high} LNCaP^{abl} cells were more sensitive to MAT2A inhibitors than the MAT2A^{low} LNCaP and 22RV1 cells.
These data support the role of MAT2A in controlling the H3K4me2 histone mark, specifically in CRPC cell models and independently of ERG. We have added a new paragraph to the Results with a description of these new data.

Reviewer #2 (Remarks to the Author):
The authors have made substantial revisions to address the Reviewer comments and have overall strengthened the manuscript. While the data are interesting and point to a novel role of MAT2A in modulating plasticity in ERG positive prostate cancer, additional prostate cancer models were not added at revision. Much of the work has been done comparing a benign cell line RWPE and one AR driven cancer cell line VCaP that does not typically develop lineage plasticity. And only ERG positive models were tested with MAT2A inhibitors. In order to propose a novel precision medicine strategy for ERG positive prostate cancer, at the very least the authors should show effects of MAT2A inhibition in ERG negative prostate cancer.

A: The reviewer disputes the suitability and robustness of the experimental models used in the study and the lack of data in ERG-negative models.

Regarding the lack of ERG-negative cell lines, notwithstanding the main focus of the manuscript on the ERG-MAT2A interaction, we also tested ERG-negative cell models and added the new data in the revised manuscript (Figure 9).
Specifically, we examined the level of MAT2A protein expression in a panel of ERG fusion-negative cell lines (Figure 9A). Interestingly, the castration-resistant LNCaP^{abl} cells had significantly higher expression of MAT2A compared to the castration-sensitive LNCaP cells (Figure 9A), supporting the notion of MAT2A upregulation also in ERG-negative CRPC. Next, we tested the effects of MAT2A inhibitors in LNCaP and LNCaP^{abl} cells as representative models of ERG-negative castration-sensitive (MAT2A^{low}) and castration-resistant counterpart (MAT2A^{high}) prostate cancer, respectively. MAT2A inhibitors significantly reduced the growth of tumor spheres of LNCaP^{abl} cells (Figure 9B), whereas LNCaP cells were minimally affected (Figure 9C). We also tested the response of 22RV1 cells. These CRPC cells are ERG-negative and have a low level of MAT2A (Figure 9A). 22RV1 cells were not affected by the MAT2A inhibitors (Figure 9D). Together, these data support the association of MAT2A with the CRPC phenotype and the vulnerability of MAT2A^{high}-expressing CRPC cells to its inhibition, even in the absence of ERG gene fusion.
Relevantly, the level of H3K4me2 histone mark correlated with MAT2A expression in these cell lines. Specifically, H3K4me2 was higher in the castration-resistant LNCaP^{abl} cells compared to LNCaP and 22RV1 cells (Figure 9E). Furthermore, inhibiting MAT2A in LNCaP^{abl} cells reduced the H3K4me2 level,

which is consistent with the specific role of MAT2A in controlling this histone modification in CRPC cells (Figure 9F).
Therefore, high MAT2A expression confers increased vulnerability to MAT2A inhibitors in various CRPC models. We addressed this point further and investigated the potential of MAT2A inhibitors in a canonical NE-CRPC model using tumor-spheroids derived ex vivo from LuCaP 145.2 xenografts. (Figure 9G). Interestingly, LuCaP 145.2 xenografts had high expression of MAT2A (Figure 9H), in line with the increased expression of MAT2A in a relevant fraction of NE-CRPC (NEPC) clinical samples. Treatment with MAT2A inhibitors reduced the growth of LuCaP 145.2 tumor spheroids (Figure 9I). Thus, these data collectively confirm the relevance of MAT2A in the evolution of CRPC with both AR-independent and NE-phenotype and the vulnerability of MAT2A^{high} CRPCs to MAT2A inhibition.

As for the number and adequacy of the experimental models, we want to point out that the data presented in the manuscript derive from experiments in multiple models, including human cell lines (VCaP, RWPE-1), patient-derived xenografts and organoids (LuCaP 35), a murine cell line and mouse-derived organoids from ERG/PTEN transgenic mice. We believe that these models with TMPRSS2:ERG gene fusion are absolutely appropriate for studying the ERG-MAT2A axis. It is not clear to us why they would not. We think that VCaP cells (the most common TMPRSS2:ERG gene fusion-positive CRPC cell line) are an appropriate model to study ERG-MAT2A cooperation. In addition to VCAP cells, the data were reproduced in TMPRSS2:ERG-positive human (LuCaP 35) and murine (ERG/PTEN) models. Furthermore, RWPE-1 cells, which lack ERG and MAT2A, were engineered to reproduce the ERG-MAT2A interaction and cooperation in a non-tumorigenic prostate epithelial cell model. We also believe that the stated inability of the ERG-positive models, VCaP and LuCaP 35, to undergo lineage plasticity is a misconception. Both models are phenotypically heterogeneous and, when challenged (e.g., androgen deprivation), exhibit enhanced lineage plasticity and undergo phenotypic transitions, gaining stemness, AR independence, and NE features. For instance, VCAP cells express, although to a low extent, NE markers like CHGA (Haffner, Bhamidipati et al. 2021). Moreover, when they transit to a castration-resistant state following extended androgen starvation, VCaP cells have higher expression of CHGA and other NE markers at RNA and protein levels. A similar process of phenotypic plasticity and phenotypic transitions is seen in PDX models, like LuCaP 35 (our group's data,unpublished).

We also want to comment on the concept of a precision medicine strategy for ERG-positive CRPC, as this might lead to misinterpretations. In this work, we examined the interaction of MAT2A and ERG in detail. We show that ERG promotes MAT2A expression, that MAT2A cooperates with ERG and other transcriptional and epigenetic factors (e.g., EZH2) to remodel the chromatin and epigenetic landscape and activate the expression of genes associated with stemness, cell plasticity, and AR-independence, and that the cooperation between MAT2A and ERG in ERG fusion-positive tumors can be disrupted very effectively by MAT2A inhibitors. In summary, we have discovered an oncogenic axis connecting ERG and MAT2A, demonstrated its impact on prostate tumorigenesis, and showed how to counteract its consequences by disrupting MAT2A-ERG interaction with inhibitors of MAT2A in ERG-positive tumors. In our opinion, these criteria fulfill the description of a precision medicine approach. Hence, the proposed strategy is based on a precisely defined mechanism linking a molecular drug target (MAT2A) to a specific feature of ERG-positive prostate cancers (ERG and MAT2A expression and functional interaction). Furthermore, in ERG-positive tumors, ERG induces MAT2A and coopts MAT2A for the epigenetic and transcriptional reprogramming of prostate cancer cells toward AR independence and castration resistance. Blocking MAT2A can prevent or reverse this process. It is conceivable that other tumors might have high MAT2A expression driven by different mechanisms and might also be responsive to MAT2A inhibition. Indeed, we show that ERG-negative CRPC models (LNCaP^{abl} and LuCap145) have high MAT2A expression and respond to MAT2A inhibitors. Nevertheless, this does not contradict the efficacy and specificity of the proposed MAT2A-based therapy for treating ERG-positive CRPC.

Specific comments:

1. As neither NEPC nor adeno models that transdifferentiate to NEPC are tested, the authors should revise statements, potentially supporting MAT2A for 'NE-initiation' (i.e. NE-transdifferentiation from CRPC-Adeno) and not for the maintenance of NEPC phenotype upon terminal differentiation.

A: We have revised the statement in the manuscript, also taking into account the new data with LuCaP 145.2, a NE-CRPC model. LuCaP 145.2 PDX has high MAT2A expression and is sensitive to MAT2A inhibitors in ex vivo tumor-spheroid assays (Figure 9G-I). These data, along with the data from clinical samples and experimental models, support the potential involvement of MAT2A in the induction and maintenance of the NE phenotype.

2. In Figure 1B and 1C, MAT2A expression levels are only slightly higher in ERG positive tumors and the scale on y-axis should be adjusted to 0-15; the authors should provide statistical analysis to support MAT2A levels being higher in ERG positive tumors. The authors mention but do not show that MAT2A expression is not correlated with expression of NE markers.

A: We have adjusted the scale of the graph axis and added the adjusted p-value (Padj=0.0014). In CRPC the level of MAT2A is significantly higher in ERGpositive samples compared to ERGnegative samples.
We confirm that we do not see correlation between MAT2A and NE markers (e.g. SYP,INSMI1) in CRPC. However,we do not think that this information is relevant.

3. Validating the findings regarding MAT2A in ERG-fusion-positive NE line, H660, and ERG-fusion-negative lines such as LNCaP, 22Rv1 is essential to develop a specific strategy for targeting MAT2A in ERG-fusion positive prostate cancer.

R: See our comment above. In the revised manuscript, we provide an assessment of MAT2A expression and MAT2A inhibitor sensitivity of ERG-negative cell lines and a NE-CRPC PDX model. High expression of MAT2A confers increased sensitivity to MAT2A inhibitors in multiple CRPC models.

4. New revised data is interesting that MAT2A+ERG over-expression led to an increase in stemness markers SOX2 and NANOG. The authors should review their RNA-Seq data from VCAP cells and confirm whether these markers (at the protein level) are downregulated upon MAT2A silencing. This is important because MAT2A may be primarily mediating stemness and not necessarily NEPC differentiation.

We agree with the reviewer that our data supports that MAT2A controls cancer stemness in multiple models. Indeed, our in vitro/ex vivo assays (i.e., tumorspheres) address this aspect, and MAT2A gain and loss of function or pharmacological inhibition influence the cancer stem-like phenotype. Furthermore, only in selected contexts, MAT2A promotes NE differentiation or maintenance of the NE phenotype.

We did not detect SOX-2 and NANOG in VCAP cells. Nevertheless, RNA sequencing revealed decreased expression of many stem cell-related genes. We followed the reviewer suggestion and using RNA sequencing data, extracted a set of cancer stem cell genes significantly downregulated following MAT2Akd in VCAP. We have added a new figure (Figure 2L) showing a set of cancer stem cell genes significantly affected by MAT2A ablation.
Stem cell markers are expected to be challenging to detect at the protein level. This is not surprising, considering that the expression of stem cell genes is restricted to the cancer stem cell subpopulation within the large and highly heterogeneous bulk population of tumor cells.

5. VCaP is not an NEPC cell line however, at the RNA-level may express low levels of INSM1, SYP, and other NE makers. Changes in these transcripts after MAT2A knockdown or overexpression should also be shown at the protein level.

A: Low expression of NE markers, such as CHGA, have been reported in VCaP cells, and these cells can undergo phenotypic transitions, also with an increased expression of NE markers as well as stemness markers. The expression of these markers is likely highly heterogeneous and low, involving only a few cells or clusters of cells, making this analysis difficult at the protein level in bulk cell cultures.

6. The authors satisfactorily addressed the comment on Figure 2 cumulative gene expression.

7. LuCaP35 is an AR-driven model that does not develop NEPC, and therefore its not clear how reduction in CHGA levels upon AG-270 impacts NE-trans-differentiation. Would also test NE model such as LuCaP 49, 93, 145, 173

See our comments above regarding the additional models that we have tested, including the NE-CRPC model LuCaP 145.2.
We are aware that the LuCaP 35 is not a canonical NE PDX. However, it is highly heterogeneous with few clusters of AR+ and NE+ cells and has the potential to evolve to castration resistance exhibiting both AR+/NE- and AR+/NE+ features (unpublished data). Thus, our data, showing a reduction of CHGA+ cell clusters in LuCaP 35 xenografts, suggest that MAT2A inhibition can block this evolution by preventing the expression of NE and stemness genes.

8. The authors should include a short paragraph on the druggability and clinical translation of MAT2A inhibitor therapy in the Discussion section.

We have added additional comments in the Discussion to emphasize the possibility of clinical translation and the feasibility of this approach.
Our data point directly to MAT2A as a driver of CRPC and an actionable therapeutic target in ERG-positive CRPC. Briefly, numerous MAT2A inhibitors are already in preclinical and clinical development. Furthermore, more compounds are in preclinical development and the field is expanding as we learn more about MAT2A and its role in methionine metabolism, epigenetics, stemness, and tumorigenesis.
At least two MAT2A inhibitors have been clinically tested for cancer patients with the MTAP gene deletion. No clinical trials have been conducted in prostate cancer patients, because of the low frequency of MTAP deletions in these patients. However, several patients with many different tumor types have been treated with these inhibitors in clinical trials. The tolerability of these drugs has been excellent, with minimal toxicity and safety issues. New upcoming trials will include dose-expansion phases also enrolling patients with MTAP-wild type tumors.
Our findings indicate that clinical testing of MAT2A inhibitors should include prostate cancer patients selected based on their genotypic/phenotypic characteristics (e.g., ERG fusion, MAT2A overexpression). This approach could be readily translated into phase I/II clinical studies with the currently available, clinically tested MAT2A inhibitors. Prospectively, ERG fusion, along with MAT2A overexpression, could be used as predictive biomarkers to select CRPC patients more likely to respond to MAT2A inhibitors in clinical studies. We also show that in ERG-positive models the inhibition of MAT2A has an immediate impact on ERG methylation and protein level, which could serve as effective and specific markers for pharmacodynamic assessment of treatment efficacy. Importantly, our data also demonstrate the synergistic effects of combinations of MAT2A inhibitors with AR antagonists and EZH2 inhibitors, which could be highly beneficial in CRPC and NE-CRPC patients to boost their clinical response.

Haffner, M. C., A. Bhamidipati, H. K. Tsai, D. M. Esopi, A. M. Vaghasia, J. Y. Low, R. A. Patel, G. Guner, M. T. Pham, N. Castagna, J. Hicks, N. Wyhs, R. Aebersold, A. M. De Marzo, W. G. Nelson, T. Guo and S. Yegnasubramanian (2021). "Phenotypic characterization of two novel cell line models of castration-resistant prostate cancer." Prostate **81**(15): 1159-1171.

Reviewer #3 (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.

Reviewer #4 (Remarks to the Author):

The authors have satisfactorily addressed most of my concerns.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
The authors have made revisions to address some of the Reviewer comments. They have included additional ERG-negative prostate cancer models and validated the efficacy of MAT2A inhibition. However, the study lacks compelling data to support a key role of MAT2A for NEPC initiation or maintenance. Given this, we recommend that the authors modify statements related to this conclusion. However, the authors might still conclude that MAT2A plays a potential role in conferring castration-resistance and could be explored as a therapeutic target.

Specific comments:

1. Though the authors did not take the Reviewer's suggestion for using NCI-H660 as an ERG-fusion positive NEPC cell line that lacks ERG and AR expression, they have used ERG-negative prostate cancer lines that strengthens their findings related to MAT2A as a potential therapeutic target for prostate cancer overall.

2. The authors have added preliminary data regarding MAT2A role in stemness. This provides opportunities to further explore MAT2A influence on stemness, and this may not necessarily be limited to NEPC transformation.

3. The authors did not show changes in NE-expression in VCaP after MAT2A knockdown/rescue or overexpression. The Haffner et. al. study that they reference showed that VCaP cells upon becoming castration-resistant maintain AR expression with no changes in CHGA and SYP expression, which may be inconsistent with NE plasticity.

4. The authors have now included LuCaP 145.2, an NEPC model and show impact of MAT2A inhibition on tumor growth, however, do not show data to support reduction in NE-markers or impact on NE-lineage.

Based on these data, it is compelling that MAT2A may be involved in conferring castration resistance but conclusions related to NE plasticity should be revised.

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General comments

We agree with the comments of the reviewer. We believe that the work provides novel and broadly relevant findings on the role of MAT2A in castration-resistant prostate cancer, mechanistic insights on the relationship of MAT2A with ERG and AR, and support testing MAT2A inhibitors for the treatment of advanced prostate cancer. We are aware that more data are needed to address the role of MAT2A in neuroendocrine prostate cancer. This aspect and the potential use of MAT2A inhibitors to prevent or reverse the neuroendocrine evolution of CRPCs will require more in-depth analyses and further studies.

Specific comments

1. Though the authors did not take the Reviewer's suggestion for using NCI-H660 as an ERG-fusion positive NEPC cell line that lacks ERG and AR expression, they have used ERG-negative prostate cancer lines that strengthen their findings related to MAT2A as a potential therapeutic target for prostate cancer overall.

In the revised manuscript, we have added data on NCI-H660 cells (Figure S9A-C). These NEPC cells express MAT2A and respond to MAT2A inhibitors (Figure S9A). Specifically, the MAT2A inhibitor AG270 reduces tumor-sphere growth (Figure S9B). Canonical NE markers, like SYP and CHGA, also were reduced in response to MAT2A inhibition (Figure S9C).

2. The authors have added preliminary data regarding MAT2A role in stemness. This provides opportunities to further explore MAT2A influence on stemness, and this may not necessarily be limited to NEPC transformation.

We agree with the reviewer's comment. Indeed, our data in multiple prostate cancer models (i.e., CRPC with and without NE features) consistently support the role of MAT2A in cancer cell stemness.

3. The authors did not show changes in NE-expression in VCaP after MAT2A knockdown/rescue or overexpression. The Haffner et. al. study that they reference showed that VCaP cells upon becoming castration-resistant maintain AR expression with no changes in CHGA and SYP expression, which may be inconsistent with NE plasticity.

In the revised manuscript we have added these data (Figure S2A and Figure S9D). Specifically, we show, by immunoblotting, that VCaP cells express the NE markers SYP and CHGA and the knockdown of MAT2A reduces their expression (Figure S2A). Moreover, treatment of VCAP cells with the MAT2Ainhibitor AG-270 reduced NE markers SYP and CHGA. These data are consistent with previous RNA-seq data analysis and with the heterogenous expression of NE markers in this CRPC model.

4. The authors have now included LuCaP 145.2, a NEPC model and show impact of MAT2A inhibition on tumor growth, however, do not show data to support reduction in NE-markers or impact on NE-lineage.

Following the reviewer's suggestion, we have included in the revised manuscript new data (Figure 9J-L) showing that MAT2A inhibition by AG-270 reduced growth of 3D organoids and concomitantly reduced the expression of the NE markers CHGA and SYP in the LuCaP 145.2 model.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
The authors have adequately addressed the experimental comments and overall the revisions have improved the manuscript. This includes validation of some of their findings in the recommended NCI-H660 models. The authors also agreed with the reviewer comments on lack of substantial evidence to support MAT2A role in NE-initiation/ maintenance and that MAT2A may be more important for cancer stemness- this could be highlighted in the discussion.

Reviewer #3:
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.

REVIEWERS' COMMENTS

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Answer: We agree and highlight this point in the discussion underlining the role of MAT2A in cancer stemness.

Reviewer #3 (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.

Answer: No comments.