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Peer Review File

PRMT1 inhibition perturbs RNA metabolism and induces DNA damage in clear cell renal cell carcinoma

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Walton et al identified PRMT1 as a critical player in renal cell carcinoma through the regulation of DNA damage response genes. Deregulation of epigenetic mechanisms is of utmost interest in carcinogenesis leading to the development of numerous inhibitors that authors used to perform their study. Even if the involvement of PRMT1 in DDR is not completely new in the field, this paper contains nice pieces of evidence of the involvement of PRMT1 in renal cell carcinoma with original approaches. The methodology sounds appropriate and data analysis, interpretation and conclusions correct. However, two aspects should be clarified before publication:
- One of the major concerns that this work raised to me is the use of solely MS023 inhibitor. Even if most of this study was conducted with MS023, I suggest to have few data with GSK3368715, another Type I PRMT inhibitor. This other potent type I PRMT inhibitor could be used at lower concentration.
- Secondly, the main conclusion of the work is that MS023 works as a critical factor for cancer growth through the activity of PRMT1. The evidence of the impact of PRMT1 on growth is obvious in Fig 3. However, in order to conclude that PRMT1 is a critical actor of renal cell carcinoma, I suggest to perform similar experiments with the 3 other PRMTs expressed in this model (PRMT3, PRMT4 and PRMT6), at least in one model. Additionally, rescue experiments using the PRMT1V1 and PRMT1V2 vector would strongly support the conclusion.

Other observations:
- Fig 1D, is it expected to see a decrease of Histone H4 expression upon MS023?
- Fig 3E, the distinction between the 3 curves is not obvious as such, even if the legend clarified it.
- In line with major comment 2, qPCR to validate the target genes expression after MS023 should be performed and compare with the inhibition of PRMT1/3/4/6 individually. The second part of this comment is not a major flaw as conclusion page 12 stand for type I PRMTs and not solely PRMT1 but would be interesting for the overall paper.
- Could the authors clarify why they analyzed BCLAF1 methylation status? Are there other interactors methylated? In line with this observation, is BCLAF1 methylation specific of PRMT1?

Minor comments:
- Fig 1B, MS094 curve is missing in 786-0 graph and should be in blue everywhere.
- Page 7, in the text it was mentioned that SGC707 is a PRMT8 inhibitor, to the contrary of Fig 1A, where it stands for PRMT3 inhibitor, a correct assignation to me.
- The Veen diagram in fig 7a should be done according to the size of the events.
- Typo in Fig 6A for MS023 concentration
- Abstract should be shortened

Reviewer #2 (Remarks to the Author):

The study by Walton et al., identified PRMT1 as a critical dependency in clear cell renal cell carcinoma (ccRCC). The authors showed that PRMT1 inhibitor MS023 had significant antitumor activity and demonstrated PRMT1 among other type I PRMTs as the key dependency in this anti-tumor activity and further demonstrated the antitumor effect of MS023 and PRMT1 knockdown in xenograph models. Mechanistically, the authors showed that MS023 affects cell cycle and DNA damage repair pathways. Then using miniTurbo-MS analysis, the authors revealed that PRMT1 interactome was rich in RNA binding proteins including the DDR mRNA metabolism regulator: BCLAF1/THRAP3 complex. Further investigation revealed impairments to DDR specific mRNA activities including nucleocytoplasmic transport and RNA splicing. This study analyzed the roles of PRMT1 in ccRCC. It is significant for ccRCC, but the roles of PRMT1 in cell death and DNA damage repair have been demonstrated extensively in the past three decades in both normal and cancer cell types. Therefore, further

mechanistic characterization of PRMT1-regulated mechanism in ccRCCs would help provide new knowledge on PRMT1 needed for a wider audience.

1. The Abstract and Introduction section comprise lengthy description of pVHL inactivation and hypoxia programs, but didn't have significant relevance to the PRMT1-regulated cell activity and gene program in this study. Can the authors either elaborate the relevance?
2. In Figure 3, overexpression of PRMT1 v1 showed a more potent effect than v2 in enhancing MS023 resistance. Why?
3. In Figure 6, the reduction of DDR protein expression, alterations of FANCD2 foci formation and DSB may be supported by PRMT1 knockdown.
4. In Figure 7C, the decline of BCLAF1 methylation indicated by anti-ASYM25 is potentially novel findings. BCLAF1 and THRAP3 have been suggested as PRMT1 substrate in a previous report without detailed mechanistic insight as to whether they are directly methylated by PRMT1 and whether arginine identified by MS are bonafide methylation sites (Nat Comm 2021). Here in ccRCCs, is PRMT1 the methyltransferase that directly methylate BCLAF1 and THRAP3? This can be assessed by in vitro methylation assay using GST-tagged proteins and in vivo methylation assays. What are the arginine sites involved? More importantly, are these two substrates critical for MS023/PRMT1 knockdown-mediated DNA damage response and cell cycle stall/cell death?
5. Figure 7D and 7E illustrated MS023-altered RNA splicing and RNA transport. How does PRMT1 regulated splicing and RNA shuttling? What are key components/substrates downstream of PRMT1 that regulates RNA splicing and shuttling? Are BCLAF1 and THRAP3 the key mediators?
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Reviewer #3 (Remarks to the Author):

In this paper Walton et al has identified MS023, an inhibitor of type I protein arginine methyltransferases (PRMTs), with ccRCC antitumor activity. Mechanistically they have revealed that MS023 treatment negatively impacts the cell cycle and DNA damage repair (DDR) pathways, while spawning an accumulation of DNA damage over time. Further data has reveled that PRMT1 plays a major role in ccRCC tumor growth and this is achieved by interacting with specific regulator of DDR mRNA metabolism: the BCLAF1/THRAP3 complex. Overall, this paper is interesting and it reveals PRMT1 as a potential attractive target in ccRCC therapy. However, there are some concerns that the authors need to address.

1- Does MS023 have an off-target effect? This comment is not referring to what has already been published (ie MS023 targeting PRMTs). My concern is that MS023 also binds to other protein(s) in cells. To resolve this issue, it would be beneficial to evaluate the MS023-biotin binding/incubation with ccRCC lysate.

2- How does MS023 treatment lead to down-regulation of genes associated with cell cycle and DDR pathways? Data presented here does not provide any mechanism towards this observation.

3- Do RCC243 cells and 786-O (data not presented here) experience a defect in cell cycle check point? The effect of MS023 on normal kidney cells have not been shown.

4- There is insufficient data presented here to support the idea of PRMT1 inhibition impacts nucleocytoplasmic transport and RNA splicing through regulation of the BCLAF/THRAP3 complex.

5- Does knockdown of PRMT1 in ccRCC phenocopies MS023 treatment? Figure 8 appears not to agree with this statement. MS023 treatment appears to slow down the rate of tumor growth whereas PRMT1 KD inhibits the rate of tumor growth.

We would like to thank the reviewers for their thoughtful and helpful comments. Please find our responses to each comment below.

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Walton et al identified PRMT1 as a critical player in renal cell carcinoma through the regulation of DNA damage response genes. Deregulation of epigenetic mechanisms is of utmost interest in carcinogenesis leading to the development of numerous inhibitors that authors used to perform their study. Even if the involvement of PRMT1 in DDR is not completely new in the field, this paper contains nice pieces of evidence of the involvement of PRMT1 in renal cell carcinoma with original approaches. The methodology sounds appropriate and data analysis, interpretation and conclusions correct.
However, two aspects should be clarified before publication:
- One of the major concerns that this work raised to me is the use of solely MS023 inhibitor. Even if most of this study was conducted with MS023, I suggest to have few data with GSK3368715, another Type I PRMT inhibitor. This other potent type I PRMT inhibitor could be used at lower concentration.

The anti-proliferative effect of GSK3368715 administration was evaluated in cell lines RCC407, RCC364, 786-0, RCC243, and RCC22. A similar inhibition profile to MS023 was noted with GSK3369715 IC_{50} values ranging from 0.7 µM to 31.4 µM noted across our cell line panel. We have now included these results in the text (page 6) and in Supplementary Figure 2, panels G and H.

- Secondly, the main conclusion of the work is that MS023 works as a critical factor for cancer growth through the activity of PRMT1. The evidence of the impact of PRMT1 on growth is obvious in Fig 3. However, in order to conclude that PRMT1 is a critical actor of renal cell carcinoma, I suggest to perform similar experiments with the 3 other PRMTs expressed in this model (PRMT3, PRMT4 and PRMT6), at least in one model. Additionally, rescue experiments using the PRMT1V1 and PRMT1V2 vector would strongly support the conclusion.

Thank you for this comment, we agree on the importance of these experiments. CRISPR knockouts of PRMT3, PRMT4 (CARM1) and PRMT6 were done in RCC243 cells and there was no effect on cell proliferation (results were the same as those obtained with control ROSA26-targeted gRNAs) as shown in Figure 2C. These results demonstrate that the only type I PRMT enzyme that displays anti-proliferative effect when knocked out in ccRCC cells is PRMT1. Additionally, no anti-proliferative effect was detected in our initial epiprobe screen (Figure 1A) with the specific type I inhibitors, MS049 (targeting PRMT4 (CARM1) & PRMT6), and SGC707 (targeting PRMT3). The rescue experiments using overexpression of PRMT1v1 and PRMT1v2 were also conducted and are shown Figure 3C-E – overexpression of PRMT1v1 rescued cells from MS023 treatment at concentrations as high as 10 µM, and PRMT1v2 also completely rescued at the 5 µM dose and partially rescued at the 10 µM dose, further confirming PRMT1 as the specific dependency responsible for growth arrest upon MS023 treatment. We have now made this clearer in the text of the manuscript.

Other observations:

- Fig 1D, is it expected to see a decrease of Histone H4 expression upon MS023?

It is not expected that MS023 will affect the levels of total H4 protein in cells, as it is only an inhibitor of the enzyme that deposits the post-translational asymmetric demethylation (aDMA) mark on H4; thus as expected, our results show the total levels of H4 are unchanged, but the aDMA mark goes down.

- Fig 3E, the distinction between the 3 curves is not obvious as such, even if the legend clarified it.

We have changed the colors on the curves to make them clearer.

- In line with major comment 2, qPCR to validate the target genes expression after MS023 should be performed and compare with the inhibition of PRMT1/3/4/6 individually. The second part of this comment is not a major flaw as conclusion page 12 stand for type I PRMTs and not solely PRMT1 but would be interesting for the overall paper.

As mentioned above, we do agree that a deeper understanding of the role of the other type 1 PRMTs that are not essential for cell proliferation (PRMT3/4/6) is of interest for future studies, but we believe this is beyond the scope of the current manuscript. We have now carried out RNAseq on cells with knockdown of specifically PRMT1 and found downregulation of mitotic and DNA repair pathways and many of the same genes as seen with MS023 treatment (Figure 4). We did not perform qPCR for validation of specific gene expression changes, as we performed Western blots for many of these genes and validated observed changes in expression at the protein level (Figure 6).

- Could the authors clarify why they analyzed BCLAF1 methylation status? Are there other interactors methylated? In line with this observation, is BCLAF1 methylation specific of PRMT1?

We selected BCLAF1 for analysis of methylation status for 2 reasons: (1) It was the highest-ranking interactor in our Bio-ID screen, and we sought to verify that our screen was indeed detecting substrates of PRMT1; (2) Due to the previously published observations that BCLAF1 and THRAP3 (which was also identified as a PRMT1 interactor in our Bio-ID experiment) are core members of a DNA damage-induced BRCA1-mRNA splicing complex that promotes the pre-mRNA splicing and subsequent transcript stability of a subset of genes involved in cellular DDR pathways[1,2]. Based on this literature, we were intrigued to investigate these two particular interactors in more detail.

We have not yet evaluated the other identified interactors for methylation but this will be a focus for future work. To our knowledge, BCLAF1 methylation by other PRMTs has not been described.

Minor comments:
- Fig 1B, MS094 curve is missing in 786-0 graph and should be in blue everywhere.
- Page 7, in the text it was mentioned that SGC707 is a PRMT8 inhibitor, to the contrary of Fig 1A, where it stands for PRMT3 inhibitor, a correct assignation to me.
- The Veen diagram in fig 7a should be done according to the size of the events.

- Typo in Fig 6A for MS023 concentration
- Abstract should be shortened

We were unfortunately not able to generate an MS094 curve at the time the experiment was carried out for technical reasons; however, the results for the remaining 5 cell lines demonstrate that, as expected, MS094 does not affect ccRCC cell proliferation. We have corrected the remaining errors. The abstract has been shortened and fits within the Nature Communications word limit.

Reviewer #2 (Remarks to the Author):

The study by Walton et al., identified PRMT1 as a critical dependency in clear cell renal cell carcinoma (ccRCC). The authors showed that PRMT1 inhibitor MS023 had significant antitumor activity and demonstrated PRMT1 among other type I PRMTs as the key dependency in this anti-tumor activity and further demonstrated the antitumor effect of MS023 and PRMT1 knockdown in xenograph models. Mechanistically, the authors showed that MS023 affects cell cycle and DNA damage repair pathways. Then using miniTurbo-MS analysis, the authors revealed that PRMT1 interactome was rich in RNA binding proteins including the DDR mRNA metabolism regulator: BCLAF1/THRAP3 complex. Further investigation revealed impairments to DDR specific mRNA activities including nucleocytoplasmic transport and RNA splicing. This study analyzed the roles of PRMT1 in ccRCC. It is significant for ccRCC, but the roles of PRMT1 in cell death and DNA damage repair have been demonstrated extensively in the past three decades in both normal and cancer cell types. Therefore, further mechanistic characterization of PRMT1-regulated mechanism in ccRCCs would help provide new knowledge on PRMT1 needed for a wider audience.

1. The Abstract and Introduction section comprise lengthy description of pVHL inactivation and hypoxia programs, but didn't have significant relevance to the PRMT1-regulated cell activity and gene program in this study. Can the authors either elaborate the relevance?

We included the description of pVHL inactivation and hypoxia programs due to their fundamental importance to the biology of ccRCC; however, we have not delved into the specific relevance of these pathways to the PRMT1-regulated gene program in this study. We have reduced the focus on this in the Abstract and have kept a single sentence including this information in the Introduction.

2. In Figure 3, overexpression of PRMT1 v1 showed a more potent effect than v2 in enhancing MS023 resistance. Why?

The variations in the N-terminal sequence of different isoforms of PRMT1 can affect their methyltransferase activity and substrate specificity[3]. Thus, the more potent ability of PRMT1v1 to rescue cells from MS023 treatment may be due to higher methyltransferase activity, or may indicate that the specific substrates of PRMT1v1 may be more important for cell proliferation in ccRCC cells. We have added a sentence to the results section about this.

3. In Figure 6, the reduction of DDR protein expression, alterations of FANCD2 foci formation and DSB may be supported by PRMT1 knockdown.

We have now carried out these assays in the presence of PRMT1 knockdown. We find that PRMT1 knockdown phenocopies many of the effects seen with MS023 treatment, including:

1) RNAseq data shows downregulation of mitotic and DNA repair pathways and many of the same genes (Figure 4)
2) DNA repair proteins (BRCA2, FANCM, FANCD2) are decreased at the protein level, FANCD2 foci formation upon mitomycin C treatment are reduced, and γH2AX foci are increased (Figure 6)

In addition, new data shows that both MS023 treatment and PRMT1 knockdown lead to accumulation of R-loops and global changes in splicing (Figure 7)

4. In Figure 7C, the decline of BCLAF1 methylation indicated by anti-ASYM25 is potentially novel findings. BCLAF1 and THRAP3 have been suggested as PRMT1 substrate in a previous report without detailed mechanistic insight as to whether they are directly methylated by PRMT1 and whether arginine identified by MS are bonafide methylation sites (Nat Comm 2021). Here in ccRCCs, is PRMT1 the methyltransferase that directly methylate BCLAF1 and THRAP3? This can be assessed by in vitro methylation assay using GST-tagged proteins and in vivo methylation assays. What are the arginine sites involved? More importantly, are these two substrates critical for MS023/PRMT1 knockdown-mediated DNA damage response and cell cycle stall/cell death?

We agree with the reviewer that these are very interesting and important questions and are a focus for follow-up to this study. However, this is a large endeavor and beyond the scope of the current manuscript. We have moved the BCLAF/THRAP3 results to the Supplementary Figures, as based on new results, we have found that MS023 treatment and PRMT1 knockdown lead to a more global effect on RNA metabolic processes, for which BCLAF1/THRAP3 function is likely only a partial explanation.

5. Figure 7D and 7E illustrated MS023-altered RNA splicing and RNA transport. How does PRMT1 regulated splicing and RNA shuttling? What are key components/substrates downstream of PRMT1 that regulates RNA splicing and shuttling? Are BCLAF1 and THRAP3 the key mediators?

Once again, we entirely agree with the reviewer that the role of altered RNA processing is a very important observation. We have now added new data to the manuscript to show that both MS023 treatment and PRMT1 knockdown lead to global splicing alterations (determined by analysis of RNAseq data) and R-loop formation (Figure 7). While we believe that BCLAF1 and THRAP3 could be participating in this phenotype, given the detection of many additional RNA-binding proteins identified as PRMT1 interactors in our Bio-ID experiment, there are likely additional RNA-binding proteins involved. We have developed a working model (Figure 9) in which PRMT1 is a master regulator of RNA metabolism, such that its inhibition leads to the accumulation of R-loops that are unresolved, causing DNA damage that then can not be repaired due to the simultaneous downregulation of expression of DDR proteins. The exact role of BCLAF1 and THRAP3, as well as other members of the PRMT1 interactome in these processes, will be the focus of future work.

6. Figure legends needs revision. For example, Figure 7d in legend described 7c in the figure.

Thank you, we have corrected these errors.

Reviewer #3 (Remarks to the Author):

In this paper Walton et al has identified MS023, an inhibitor of type I protein arginine methyltransferases (PRMTs), with ccRCC antitumor activity. Mechanistically they have revealed that MS023 treatment negatively impacts the cell cycle and DNA damage repair (DDR) pathways, while spawning an accumulation of DNA damage over time. Further data has reveled that PRMT1 plays a major role in ccRCC tumor growth and this is achieved by interacting with specific regulator of DDR mRNA metabolism: the BCLAF1/THRAP3 complex. Overall, this paper is interesting and it reveals PRMT1 as a potential attractive target in ccRCC therapy. However, there are some concerns that the authors need to address.

1- Does MS023 have an off-target effect? This comment is not referring to what has already been published (ie MS023 targeting PRMTs). My concern is that MS023 also binds to other protein(s) in cells. To resolve this issue, it would be beneficial to evaluate the MS023-biotin binding/incubation with ccRCC lysate.

A rigorous chemo-proteomics study was carried out by Scheer et al[4] where IP-mass-spec with biotinylated MS023 in was done in HEK293 embryonic kidney cells. This showed engagement of the expected PRMT1, 3, 4 and 6, as well as their respective binding partners, demonstrating the selectivity of this probe. In addition, while MS023 does bind to other proteins inside the cell, as shown in the above-mentioned manuscript, the phenotype specificity that we observed with PRMT1 knockout but not the other PRMTs, as well as the fact that the phenotype could be rescued through overexpression of PRMT1 isoforms, indicates that the observed phenotypes are specific to MS023's on-target effects. Given the extent and rigor of previous studies in this area, we consider that such a chemo-proteomics study specifically in ccRCC cells is beyond the scope of the current manuscript.

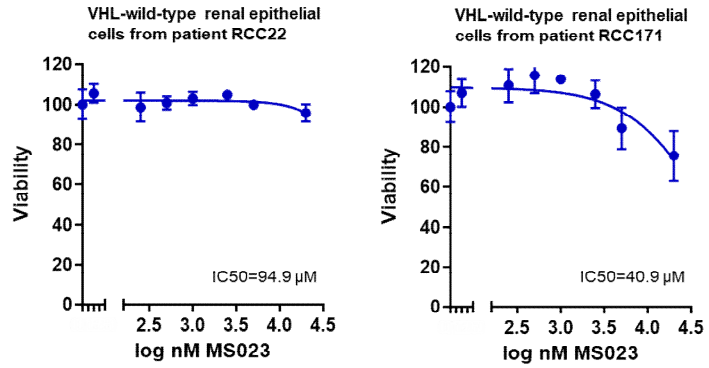
2- How does MS023 treatment lead to down-regulation of genes associated with cell cycle and DDR pathways? Data presented here does not provide any mechanism towards this observation.

We hypothesize that MS023 treatment leads to DNA damage that goes unrepaired, as demonstrated by accumulation of γH2AX foci over time. This leads to cell cycle arrest, which results in global downregulation of genes associated with the cell cycle. We do not yet understand the mechanism for the decrease in DDR gene transcription, but it was this observation that led us to prioritize BCLAF1/THRAP3 as candidates for follow-up due to previous reports that this complex is specifically involved in the splicing and nuclear export of DDR transcripts, and we do see some evidence of this (Supp. Fig. 9). However, there are likely additional mechanisms involved that remain to be elucidated, such as changes in transcription that are regulated by the H4R3me2a mark. Understanding this will be a major focus of future work.

3- Do RCC243 cells and 786-O (data not presented here) experience a defect in cell cycle check point? The effect of MS023 on normal kidney cells have not been shown.

We have not investigated specifically whether RCC243 and/or 786-0 have inherently defective cell cycle checkpoints. We carried out MS023 treatments on two normal (VHL-wild-type) kidney epithelial cell lines isolated from two ccRCC patients (see below) which proved to be more

resistant to MS023 treatment than our VHL-mutant ccRCC cell lines, indicating that there are potential differences between ccRCC cells and normal renal epithelial cells in their MS023 response. We have not included these results in the manuscript, as they are preliminary, and more experiments are needed.



In addition, the drug was well tolerated at doses used in mice. These data suggest a potential therapeutic window. However, more toxicity work, dose optimization and drug combination experiments need to be done. This is a major focus of current work in our lab.

4- There is insufficient data presented here to support the idea of PRMT1 inhibition impacts nucleocytoplasmic transport and RNA splicing through regulation of the BCLAF/THRAP3 complex.

We agree with this comment; while we do demonstrate that BCLAF1 is a PRMT1 substrate and see some trends toward changes in nucleocytoplasmic transport, further studies are needed to gain a better understanding of the role of BCLAF1/THRAP3 in mediating the observed responses to MS023 treatment. We have moved this data to the supplementary figures and included some new results showing that both MS023 treatment and PRMT1 knockdown lead to broader defects in RNA metabolism, as indicated by global changes in splicing and the formation of R-loops under both conditions.

5- Does knockdown of PRMT1 in ccRCC phenocopies MS023 treatment? Figure 8 appears not to agree with this statement. MS023 treatment appears to slow down the rate of tumor growth whereas PRMT1 KD inhibits the rate of tumor growth.

In addition to the proliferation assay and decreased aDMA that we showed before, we have now repeated many of the experiments previously done with MS023 treatment using PRMT1 knockdown. We find that PRMT1 knockdown phenocopies many of the effects seen with MS023 treatment, including:

1) RNAseq data shows downregulation of mitotic and DNA repair pathways and many of the same genes (Figure 4)
2) DNA repair proteins (BRCA2, FANCM, FANCD2) are decreased at the protein level, FANCD2 foci formation upon mitomycin C treatment are reduced, and γH2AX foci are increased (Figure 6)

In addition, new data shows that both MS023 treatment and PRMT1 knockdown lead to accumulation of R-loops and global changes in splicing (Figure 7)

A likely explanation for the results seen in Figure 8, where the tumor growth inhibition is less striking for MS023 treatment *vs* PRMT1 knockdown, is that due to selection, every cell contains the knockdown construct, and knockdown is continuous. For *in vivo* drug treatments, the efficiency of PRMT1 targeting may be less due to imperfect penetrance of the drug to all tumor cells, and the fluctuating drug concentrations throughout the treatment period. MS023 is a tool compound and has not been optimized for *in vivo* applications; drug optimization would likely be required to move type 1 PRMT (or PRMT1)-targeted drugs into the clinical setting.

References:

1. Savage, K. I. *et al.* Identification of a BRCA1-mRNA Splicing Complex Required for Efficient DNA Repair and Maintenance of Genomic Stability. *Mol. Cell* **54**, 445–459 (2014).
2. Vohhodina, J. *et al.* The RNA processing factors THRAP3 and BCLAF1 promote the DNA damage response through selective mRNA splicing and nuclear export. *Nucleic Acids Res.* **45**, 12816–12833 (2017).
3. Thiebaut, C. *et al*. Structure, activity, and function of PRMT1. *Life* 11, 1147 (2021).
4. Scheer, S. *et al*. A chemical biology toolbox to study protein methyltransferases and epigenetic signaling. *Nat. Commun.* 10:19 (2019).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Authors answered to most of my comments, except to one aspect.
In Fig 1D left panel, there is a decrease of histone H4 upon MS023 treatment in 786-0. As expected, and detailed by authors in their rebuttal letter, only ADMA should decrease. Please explain this aspect.

Reviewer #2 (Remarks to the Author):

The revised manuscript improved on molecular mechanistic analysis of how MS023 treatment/PRMT1 knockdown impact ccRCC. But there is concerns that needs to be addressed over new figures included.

1. New figure 7C is concerning, since R-loop staining using S9.6 normally appear as punctate staining, not a perfused pattern.
2. Bar graphs at the bottom of new figure 7D illustrating alternative splicing needs clarification as to which groups were compared to yield the bar graph.
3. It is also recommended to move Supplemental figure 9 to the official figures as it represents part of the novelty in this article.

Reviewer #3 (Remarks to the Author):

The authors have made a very good job addressing my questions and concerns by logical explanations and or addition of new data. This manuscript is acceptable for publication in Nature Communications.

We would like to thank the reviewers for their thoughtful and helpful comments on the revised version of our manuscript. Please find our responses to each comment below.

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Authors answered to most of my comments, except to one aspect. In Fig 1D left panel, there is a decrease of histone H4 upon MS023 treatment in 786-0. As expected, and detailed by authors in their rebuttal letter, only ADMA should decrease. Please explain this aspect.

We apologize for not being clear. The Western blot analysis for H4R3me2a, shown in Fig. 1D, assesses changes in H4R3me2a in response to MS023 treatment. The inclusion of histone H4 in this Western blot serves as a gel loading control, which is common practice when blotting for histone post-translational modifications, as referenced in previous studies[1-3]. Variations in the H4 signal, as noted by the reviewer, do not reflect decreases in histone H4 expression, but rather variations in the amount of purified histones loaded on the blot. We have added explicit language to the figure caption to clarify this.

References:

[1]Eram, M. S. *et al.* A Potent, Selective, and Cell-Active Inhibitor of Human Type I Protein Arginine Methyltransferases. *ACS Chem Biol* **11**, 772-781, doi:10.1021/acschembio.5b00839 (2016).

[2]Yao, B. *et al.* PRMT1-mediated H4R3me2a recruits SMARCA4 to promote colorectal cancer progression by enhancing EGFR signaling. *Genome Med* **13**, 58, doi:10.1186/s13073-021-00871-5 (2021).

[3]Yu, W. *et al.* Histone tyrosine sulfation by SULT1B1 regulates H4R3me2a and gene transcription. *Nat Chem Biol* **19**, 855-864, doi:10.1038/s41589-023-01267-9 (2023).

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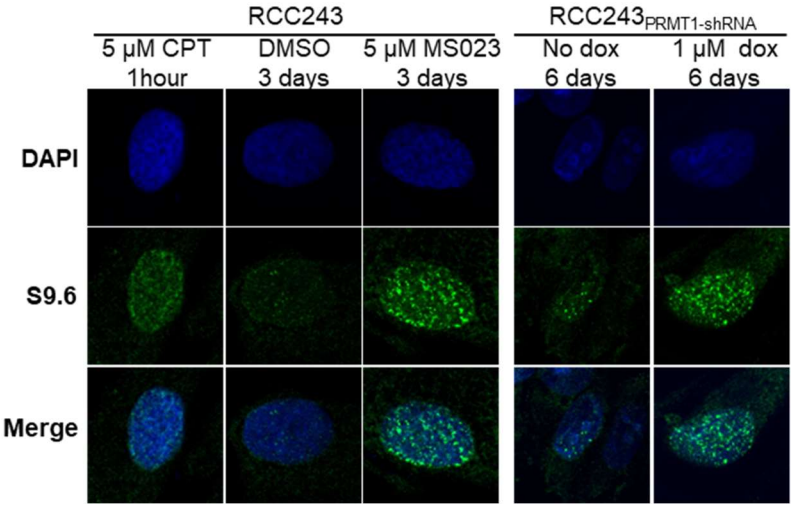
1. New figure 7C is concerning, since R-loop staining using S9.6 normally appear as punctate staining, not a perfused pattern.

Thank you for your comment. The representative images from the R-loop staining and quantification experiment were captured using a standard fluorescence microscope. Consequently, the punctate nature of the staining may appear subdued due to the flattening and overlapping of features in these 2D images. Given that our quantification method relies on measuring total nuclear intensity, as reported in many other manuscripts[4-6], we did not initially take 3D confocal images. However, to more clearly visualize the punctate characteristics of the staining, we re-imaged our slides using a 3D confocal microscope (Zeiss LSM710), and the punctate staining pattern was more apparent (see representative images below). We would prefer to maintain the 2D images in the manuscript, since they are representative of the images used for the quantitative analysis that was performed.



References:

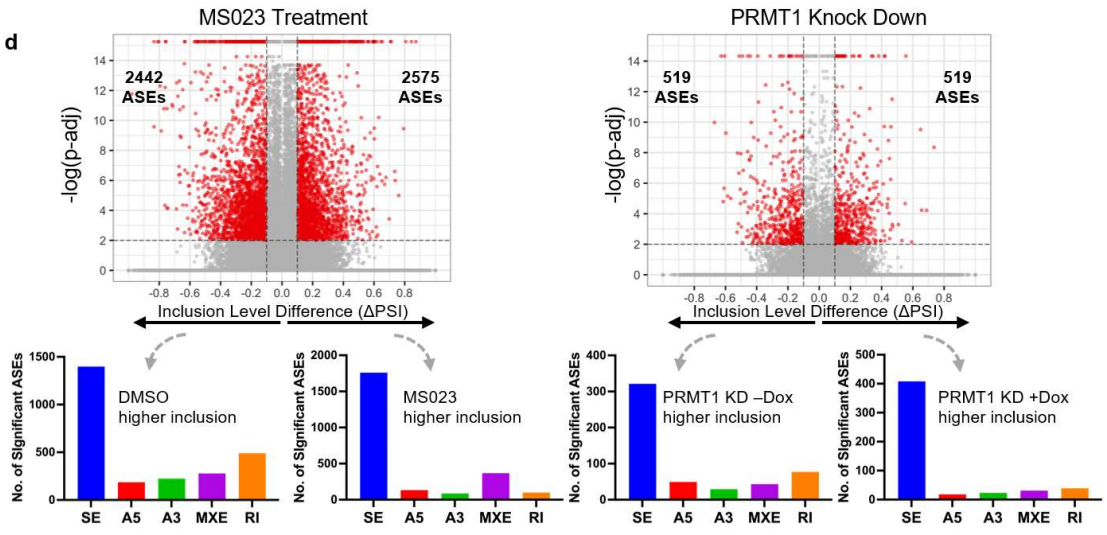
[4]Bhattacharjee, R. *et al.* Compromised transcription-mRNA export factor THOC2 causes R-loop accumulation, DNA damage and adverse neurodevelopment. *Nat Commun* **15**, 1210, doi:10.1038/s41467-024-45121-5 (2024).

[5]Jayakumar, S. *et al.* PSIP1/LEDGF reduces R-loops at transcription sites to maintain genome integrity. *Nat Commun* **15**, 361, doi:10.1038/s41467-023-44544-w (2024).

[6]Wang, Y. *et al.* ZFP281-BRCA2 prevents R-loop accumulation during DNA replication. *Nat Commun* **13**, 3493, doi:10.1038/s41467-022-31211-9 (2022).

2. Bar graphs at the bottom of new figure 7D illustrating alternative splicing needs clarification as to which groups were compared to yield the bar graph.

Apologies for the confusion. The bar graphs are meant to reflect the volcano plots that they are underneath, which are showing the alternative splice events (ASEs) found in DMSO vs MS023-treated cells (on the left) or the PRMT1 knockdown, no dox vs + dox, on the right. We have clarified this in the figure legend, and added arrows to the figures to make it more clear. The modified Figure 7d is shown below:



3. It is also recommended to move Supplemental figure 9 to the official figures as it represents part of the novelty in this article.

We agree with the reviewer that the role of BCLAF1 and THRAP3 in the observed phenotype are novel and important findings, and while we've demonstrated that BCLAF is a PRMT1 substrate and that some trends toward changes in nucleocytoplasmic transport exist, further studies are needed to gain a better understanding. As such, we would advocate for leaving these results in the supplementary figures and instead highlighting the new data added to the manuscript that shows both MS023 treatment and PRMT1 knockdown lead to global splicing alterations (determined by analysis of RNAseq data) and R-loop formation (Figure 7). The exact role of BCLAF1 and THRAP3, as well as other members of the PRMT1 interactome in these processes, is the current focus of ongoing work in our lab.

Reviewer #3 (Remarks to the Author):

The authors have made a very good job addressing my questions and concerns by logical explanations and or addition of new data. This manuscript is acceptable for publication in Nature Communications.

We thank the reviewer for their time and helpful comments.