EMBO reports

Arginine methylation-dependent LSD1 stability promotes invasion and metastasis of breast cancer

Jiwei Liu, Jingxin Feng, Lili Li, Luyao Lin, Jiafei Ji, Cong Lin, Lingxia Liu, Na Zhang, Dandan Duan, Zhongwei Li, Baiqu Huang, Yu Zhang and Jun Lu

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Editor: Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 June 2019

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, I will not further detail them here, also as I feel that all points need to be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are

highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study are deposited in an appropriate public database. See:
http://www.embopress.org/page/journal/14693178/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate

source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please have the manuscript proofread by a native speaker and organise the study better to improve legibility (see also the report of referee #1, and the last point of referee #3). Our publisher also offers a manuscript editing service: https://wileyeditingservices.com/en/english-language-editing/

10) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The article "Arginine methylation-dependent LSDI stability promotes invasion and metastasis of breast cancer" by Liu et al. highlights how PRMT4 dimethylates LSD1 and maintains its stability via USP7 recruitment.

In this manuscript, the authors identified the solo methylation site of LSD by substitution of four arginine residues with alanine and they demonstrated, by in vitro methylation analysis, that only R838A abolished PRMT4 mediated methylation of LSD1. Moreover, they showed that LSD1 methylation leads to decreased ubiquitination meditated by USP7 and therefore stabilises LSD1. These findings may be of interest and relevance to understand the interplay between post-translational modifications and precisely how the PRMT4-USP7-LSD1 axis is involved in breast carcinogenesis.

Although, it has been reported in 2015 by another group that LSD1 is lysine trimethylated by methyltransferase SUV39H2 on K322 suppressing its polyubiquitination and subsequent degradation, Liu and colleagues demonstrates for the first time that LSD1 is a new substrate of CARM1. The originality of this paper comes from the generation of a polyclonal antibody specifically recognizing LSD1 R838me2a where its specificity was validated by many techniques. The authors provide enough evidence to prove and justify their conclusions using shPRMT4 and inhibitors in an "in vitro and in vivo" context. In my opinion, this work is of potential interest, the

methodological details are convincing which does provide confidence that the work is technically sound.

Minor/specific points:
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• page 3: "To date, however"; use one of them
• page 4 first paragraph: "However, if the stability... is far well understood": not clear
• page 15: "Recently studies have shown that LSD1 and PRMT4 co-existence in the same complex".
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- For the in vivo study, I wonder why they just injected the 2 mutants LSD1 R838A and R838F knowing that the F mutant maybe a constitutive methylated mimetic and showed high mobility in vitro. It will be more pertinent to inject the LSD1 R-K mutants as well to show how it will affect the breast metastasis in vivo.

- It would be nice to compare MDA-MB-231 characterized by a high level of PRMT4 and LSD1 methylation with MCF10 which is barely expressing to see if the ubiquitination and the stabilisation of LSD1 (in vitro mechanism) show similar effect as the shPrmt4 or the inhibitor MS049.

- Page 13, Figure 5, I assume that the authors wanted to say "Beside, MS049 or P5091 treatment led to...with a notable increase in H3K4me2...Meanwhile, the enrichment of LSD1 at vimentine...accompagnied by an increase of H3K9me2. They wrote "decrease" in the text and they are showing an increase in the figure.

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- Review page 20: Materials and Methods: "wash the beads..., resuspended in 40-60µL and not 40-60ml of 2x loading buffer).

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- PRMT4 could be replaced by CARM1 in all the manuscript.

- Add the protein ladders for all westerns.

Referee #2:

In this MS titled "Arginine methylation-dependent LSD1 stability promotes invasion and metastasis of breast cancer", the authors found that PRMT4 can methylate LSD1 at R 838 site, which enhance its stability via recruitment of deubiquitinating enzyme USP7. Methylated LSD1 gains ability to promote cell migration and lung metastasis. In general, the identification of LSD1 methylation as a new mechanism for cancer metastasis agrees with the known function of PRMT4 in promoting metastasis. The new mechanism is very interesting and overall the conclusion is supported by their data. However, there are still some issues needed to be addressed.

Major comments:
1) Fig. 1E, the in intro methylation assays results are not so convincing. The bands seem to be cut-off from a gel and look fuzzy.
2) Fig.3A, USP 9x, 28, 7 and 22 have the similar function in deubiquitination and all of them interact with LSD1 WT. The authors should knockdown individual one and measure the stability of LSD1. The endogenous protein expression levels should be measured by western blot.
3) The authors showed results on methyl-LSD1 in migration and invasion. It is important also characterize cell proliferation.
4) MCF710A is a non-tumorigeneic mammary epithelial cells. The use of this cell in migration assay in Figure 4C and D is not justified. MCF7 or other breast cancer cell lines should be used.

Minor comments:
1) Original MDA-MB-231 is in general not very metastatic, only the selected clone LM2 is metastatic. It is surprising that massive lung metastasis could be observed in Fig. 4E, F. The authors should validate this cell line by STR analysis.
2) The different mechanism of LSD1 regulating E-cadherin and vimentin is not convincing. Fig.5 should characterize the difference of occupancy of H3K4me2 and H3K9me2 on these two gene promoters.

Referee #3:

This manuscript describes that LSD1 is methylated at R838 by arginine methyltransferase PRMT4 and this methylation enhances LSD1 protein stability. The authors found that the mutation of certain amino acid residues in the region around R838 motif reduces the binding of USP7, a deubiquitylase, which is previously shown to regulate LSD1 protein stability. While some parts of experiments are well done, there are several major problems on some critical data in the manuscript:

1) Methylation and methylation antibodies are not well characterized. Although R838 in LSD1 appears to be a good arginine substrate for PRMT4, as it has the consensus "L/I-P-R" methylation site conserved in other PRMT4 substrates, the prove is not very convincing. In Figure 1A, the mass spectrometry of the peptide 825-838 from of transfected LSD1 has no molecular weight indicating whether it is mono- or di-methylated, or not methylated. In Figure 1E, it seems that not only R838A but also 4RA, R608A, are reduced in the methylation assays by GST-PRMT4. R108A seemed to stimulate LSD1 methylation. There is no explanation in the text. In Figure EV2B, the test of methylation and non-methylation antibodies, how was this experiment done? How can one use the same blot to incubate it with two antibodies (Nme-ab and me-ab)? There is no description on how the me-ab is raised, purified, and blotted.

2) Ubiquitination assays in Figure 2H and I were not correctly conducted so it is not convincing. Since anti-LSD1 immunoprecipitation could bring down many LSD1-associated proteins that can be polyubiquitinated which can be detected by anti-Ub antibody, this data is also not convincing.

3) How R838 methylation affects USP7 remains not very well characterized. The major significance of R838 by PRMT4 is that mutation of this LPR motif reduced USP7 binding in transfected cells. Previous studies (Oncology reports 36, 29935-2945, 2016) showed USP7 binds to LSD1 but did not map the binding site on LSD1. Thus, a characterization of USP7 binding to LSD1 both in vivo and in vitro would address why methylated R838 interferes with binding of USP7.

This manuscript is also poorly written and figures are poorly labeled so it is very difficult to read. Some figures and text do not match, such as in Figure 1EV1D, the text states that "LSD1 was markedly increased after PRMT4 overexpression in HEK-293T cells". However, the figure showed LSD1 is markedly reduced. Overall, the finding is potentially interesting and may be important for the regulation of LSD1 protein levels. However, many critical experiments are not convincing.

Referee #1:

The article "Arginine methylation-dependent LSDI stability promotes invasion and metastasis of breast cancer" by Liu et al. highlights how PRMT4 dimethylates LSD1 and maintains its stability via USP7 recruitment.

In this manuscript, the authors identified the solo methylation site of LSD by substitution of four arginine residues with alanine and they demonstrated, by in vitro methylation analysis, that only R838A abolished PRMT4 mediated methylation of LSD1. Moreover, they showed that LSD1 methylation leads to decreased ubiquitination meditated by USP7 and therefore stabilises LSD1. These findings may be of interest and relevance to understand the interplay between post-translational modifications and precisely how the PRMT4-USP7-LSD1 axis is involved in breast carcinogenesis.

Although, it has been reported in 2015 by another group that LSD1 is lysine trimethylated by methyltransferase SUV39H2 on K322 suppressing its polyubiquitination and subsequent degradation, Liu and colleagues demonstrates for the first time that LSD1 is a new substrate of CARM1. The originality of this paper comes from the generation of a polyclonal antibody specifically recognizing LSD1 R838me2a where its specificity was validated by many techniques. The authors provide enough evidence to prove and justify their conclusions using shPRMT4 and inhibitors in an "in vitro and in vivo" context. In my opinion, this work is of potential interest, the methodological details are convincing which does provide confidence that the work is technically sound.

Response:

We are very grateful for your critical reading of the MS, and for the positive comments towards our work, which we regard as a big impetus and encouragement to our future work.

Minor/specific points:

Q1: The general English used in this manuscript is poor and needs to be proof-read and improved. Few examples to review:

- page 3: "To date, however"; use one of them
- page 4 first paragraph: "However, if the stability... is far well understood": not clear
- page 15: "Recently studies have shown that LSD1 and PRMT4 co-existence in the same complex".
- Page 16: "These data implicate that the alternative splicing of LSD1 can be a means??"

Response:

Thank you for pointing out the specific mistakes. We have corrected these faults accordingly in the revised manuscript. We have also managed to improve and refine the English language in the text by careful proof-reading and by consulting native English speakers. We hope it now becomes more comfortable to read.

Q2: In figure 1E, the authors mentioned that both GST-LSD1 4RA and R838A show less methylation, however the autoradiography film did not reflect what they said. The figure needs to be replaced by another that is more convincing.

Response: We are sorry for the unsatisfactory quality of the figure. We have now repeated this experiment and replaced this figure with a more convincing image, which is more indicative that LSD1 R838 is the sole methylation site methylated by CARM1 (new Fig 1E, see below).

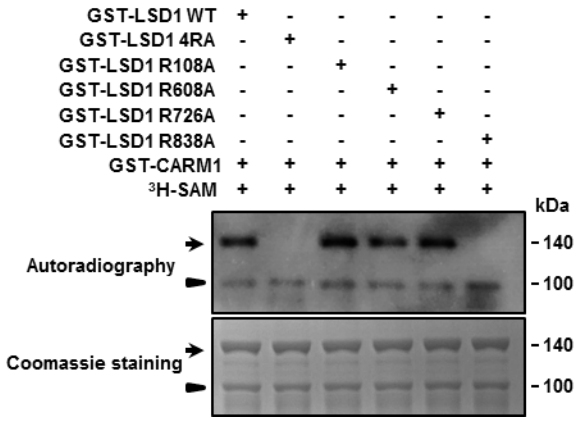


Fig 1E. *In vitro* methylation assays. Methylated proteins were detected via autoradiography.

Moreover, we have analyzed the methylation of LSD1 by immunoblotting with anti-ASYM after incubating GST-tagged PRMT4 with GST-tagged LSD1 WT, or its mutants (R108A, R608A, R726A, R838A, 4RA), in the presence of SAM. Notably, the R838A, as well as 4RA, abolished PRMT4-mediated methylation of LSD1, which we think may further strengthen our conclusion that LSD1 is methylated by CARM1 at R838. This additional result is now presented in Fig EV1F in the revised manuscript.

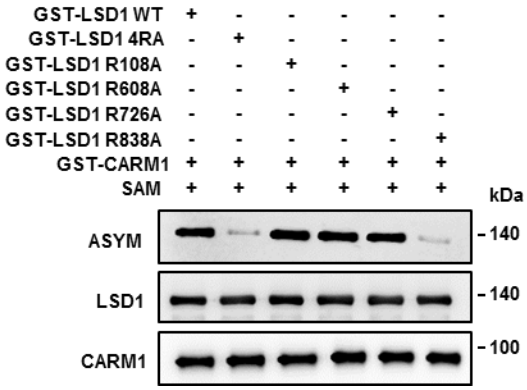


Fig EV1F. *In vitro* methylation assays. Methylation of LSD1 protein was analyzed by Western blotting.

Q3: MS049 is known to be a dual inhibitor for PRMT4 and PRMT6 (Shen Y et al., 2016). Why they did not use another more specific inhibitor such as: EZM2302 (GSK3359088) or TP064. Also, they used a high concentration of MS049 (30µM) to see a good inhibition of the methylation, knowing that that the IC50: 34 nM for PRMT4; 43 nM for PRMT6??? (at 5 µM, the effect is not optimal; check figure 2D).

Response: Yes, the reviewer is right that MS049 is an inhibitor for both PRMT4 and PRMT6. The reason we used MS049 was that when we started this study 3 years ago, the more specific inhibitors of PRMT4 such as EZM2302 (Allison E. Drew et al., 2017) and TP064 (Kazuhide Nakayama et al., 2018) had not been identified and commercially available, and MS049 was the most suitable inhibitor of PRMT4 we could pick up at that time. In addition, we decided to use MS049 because only PRMT4, not PRMT6, can methylate LSD1 (Fig EV1C), while MS049 can effectively inhibit the methylation of LSD1. It is for these reasons that we believe the inhibitory activity of MS049 against PRMT4 may meet the needs under our experimental system. Considering the consistency of experimental conditions, MS049 was used throughout our experiments.

As for the concentration, Shen Y et al. (J Med Chem, 2016) reported that MS049 partially decreased Med12-Rme2a level in HEK293 cells at 3 µM, whereas it displayed a good inhibition at 9 µM in which Med12-Rme2a level disappeared (see their Fig. 4B). Actually, in our assays we found that

MS049 moderately reduced LSD1 R838me2a level in MDA-MB-231 cells at 5 μM, while the LSD1 level showed a negligible change. When the concentration of MS049 was raised up to 30 μM, the LSD1 R838me2a level disappeared, and LSD1 was also significantly decreased, which is consistent with the results of our PRMT4 knockdown results in MDA-MB-231 cells (as seen in Fig 2B). So we chose 30 μM as the working concentration in our system.

It is worth mentioning that we treated HEK-293T cells with various amounts of MS049, and observed a good inhibition of the global asymmetric methylation and LSD1 R838me2a levels at concentration of 5 μM (Figure R1A for referee), which is consistent with Shen's report. Meanwhile, the global asymmetric methylation level of MDA-MB-231 cells was examined under MS049 treatment, and we observed a sharp decrease at 30 μM (Figure R1B for referee), which was similar to that of 5-15 μM in HEK-293T cells. However, it seems that the MDA-MB-231 triple negative breast cancer cells are much less sensitive to MS049 than HEK-293T cells, probably due to the influence of the complicated background caused by dysregulated proteins such as PRMT4 in MDA-MB-231 cells.

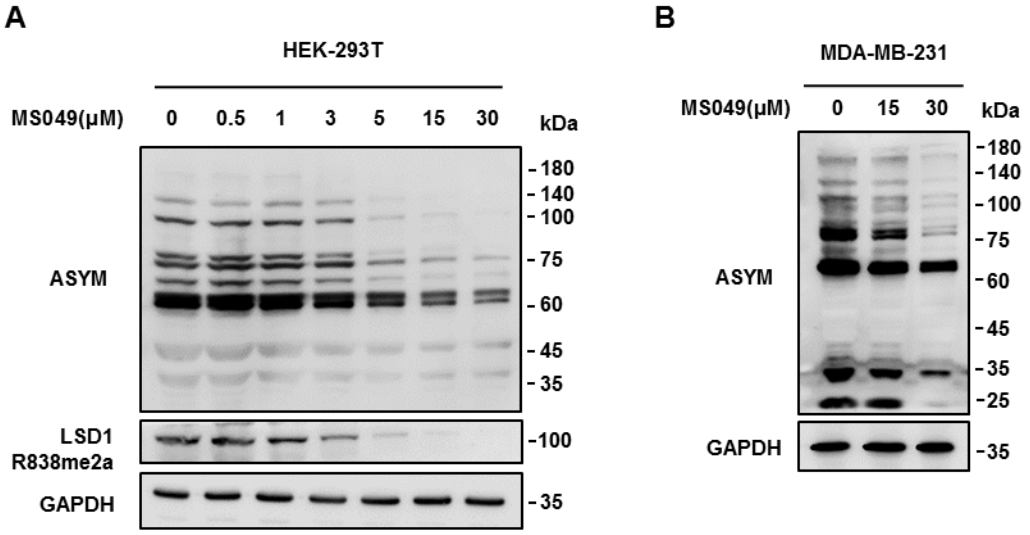


Figure R1. HEK-293T (A) and MDA-MB-231 (B) cells were treated with indicated amounts of MS049, the global asymmetric methylation status were examined by western blotting.

Q4: For the in vivo study, I wonder why they just injected the 2 mutants LSD1 R838A and R838F knowing that the F mutant maybe a constitutive methylated mimetic and showed high mobility in vitro. It will be more pertinent to inject the LSD1 R-K mutants as well to show how it will affect the breast metastasis in vivo.

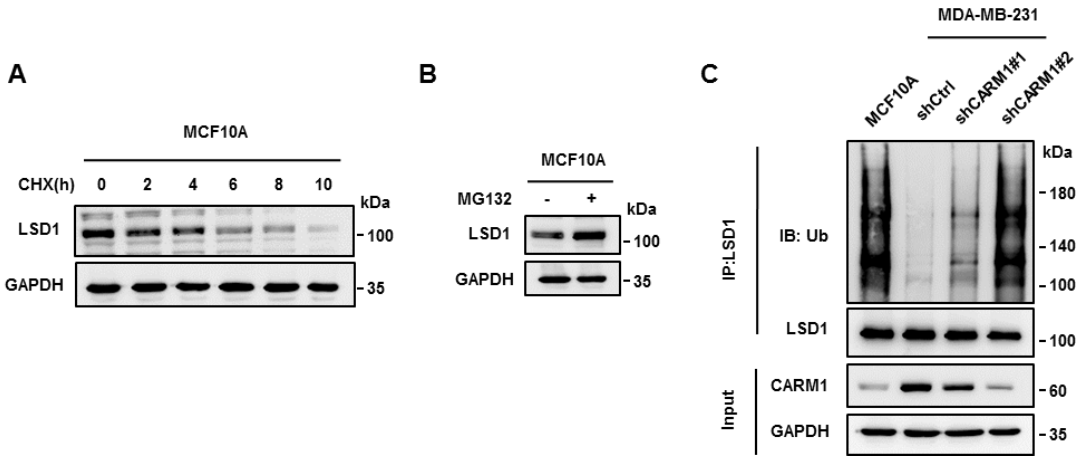
Response: We agree with the referee and performed this experiment as the referee suggested. In details, we injected LSD1 R838K mutant and LSD1 WT and the other two mutants LSD1 R838A and LSD1 R838F, into the tail veins of female nude mice. The results showed that mice injected with MM-231-shLSD1 R838K/A cells formed fewer lung metastasis foci than that injected with MM-231-shLSD1-WT or MM-231-shLSD1-LSD1 R838F cells. These data have been incorporated into Results, and the relevant section of text has been re-written in the revised manuscript (new Fig 4E-F).

Q5: It would be nice to compare MDA-MB-231 characterized by a high level of PRMT4 and LSD1 methylation with MCF10 which is barely expressing to see if the ubiquitination and the stabilisation of LSD1 (in vitro mechanism) show similar effect as the shPrmt4 or the inhibitor MS049.

Response: We are grateful to the referee for this very helpful suggestion. In order to examine the ubiquitination and stabilization of LSD1 in MCF10A cells, we treated MCF10A cells with protein synthesis inhibitor cycloheximide (CHX) and found that the half-life of LSD1 in MCF10A was

much shorter than that of MDA-MB-231 cells, which was similar to that of PRMT4-knockdown MDA-MB-231 cells (seen Fig 2G and new Appendix Fig S2A).

As stated in the text, LSD1 is degraded through ubiquitin-proteasome pathway, and we also observed a robust increase of LSD1 upon the addition of proteasome inhibitor MG132 in MCF10A cells (new Appendix Fig S2B). We then performed immunoprecipitation with anti-LSD1 to explore the ubiquitination level of endogenous LSD1 in MCF10A and PRMT4-knockdown MDA-MB-231 cells after treated with MG132 for 10 h. As a result, the ubiquitination of LSD1 in MCF10A was much stronger than that of MDA-MB-231 cells, similar to that of MDA-MB-231-shPRMT4#2 cells (new Appendix Fig S2C), which has higher PRMT4 knockdown efficiency. Collectively, these results further strengthen our conclusion that the PRMT4-mediated methylation of LSD1 promotes LSD1 stabilization. Relevant new results have been presented in Appendix Fig S2 and Result 2.



Appendix Fig S2. Ubiquitination and stabilization of LSD1 in MCF10A cells.

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Response: We apologize for the mistakes. We have corrected it accordingly in the revised manuscript.

Q7: Page 17, they reported that USP7 plays a role in tumor progression by stabilizing a number of substrates (PHF8, Ki-67), I wonder if the authors checked the status/level of these factors after PRMT4 inhibition.

Response: It is indeed a good question. As stated in the text, our research focuses on exploring the mechanisms by which dysregulation of LSD1 promotes breast cancer progression, so we did not examine the levels of these two factors in our experiments. However, we do not exclude the possibility that they might play a role in promoting breast cancer progression, and this has proposed a good scientific issue for our future work.

Q8: Review page 20: Materials and Methods: "wash the beads..., resuspended in 40-60µL and not 40-60ml of 2x loading buffer).

Response: Thank you for pointing out the mistakes. We have corrected these mistakes accordingly.

Q9: The authors can check the effect of depletion or inhibition of PRMT4/CARM1 by showing its effect on the global asymmetric and/or symmetric methylation using commercial antibodies.

Response: We thank the referee for this suggestion. As suggested, we performed Western blotting with anti-ASYM to probe the global asymmetric dimethylation status after PRMT4 inhibition.

Specifically, we treated MDA-MB-231 cells with indicated amounts of PRMT4 inhibitor MS049 for 48 h, and we found that the global asymmetric dimethylation was significantly reduced at concentration of 30 μM and also decreased in a certain degree at 15 μM. These results have been shown in **Q3** (seen Figure R1B for referee).

Q10: PRMT4 could be replaced by CARM1 in all the manuscript.
Response: Thank you for your suggestion. We have replaced PRMT4 with CARM1 in the revised manuscript.

Q11: Add the protein ladders for all westerns.
Response: Following the referee's suggestion, we have added the protein ladders for all Western blots.

Referee #2:

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Major comments:

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Response: We are sorry for the unsatisfactory quality of the image. As a remedy, we have repeated this experiment and replaced this figure with a new one of better quality, which indicates that LSD1 R838 is the sole methylation site methylated by PRMT4 (CARM1) (new Fig 1E), and we hope the new figure is satisfactory.

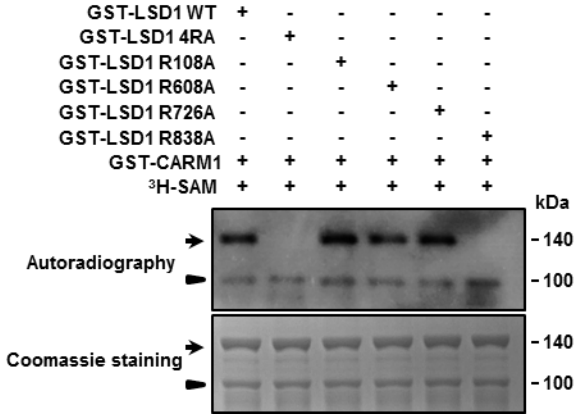
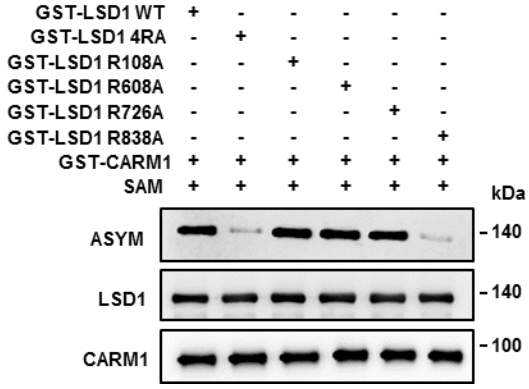


Fig 1E. *In vitro* methylation assays. Methylated proteins were detected via autoradiography.

Moreover, we also analyzed the methylation of LSD1 by immunoblotting with anti-ASYM after incubating GST-tagged PRMT4 (CARM1) with GST-tagged LSD1 WT or its mutants (R108A, R608A, R726A, R838A, 4RA) in the presence of SAM. Notably, the R838A as well as 4RA, abolished PRMT4-mediated methylation of LSD1 which further strengthened our conclusion (Fig EV1F).



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Q2: Fig.3A, USP 9x, 28, 7 and 22 have the similar function in deubiquitination and all of them interact with LSD1 WT. The authors should knockdown individual one and measure the stability of LSD1. The endogenous protein expression levels should be measured by western blot.

Response: We thank the referee for this constructive suggestion. Accordingly, we interfered USP9X, USP28 and USP22 in MDA-MB-231 cells by using shRNAs, respectively, and found that LSD1 protein levels exhibited negligible changes irrespective of the knockdown of each of these deubiquitylases (Figure R2 for referee). However, our previous results showed that USP7 knockdown downregulated endogenous LSD1 (see Fig EV4A), suggesting that only USP7 play a role in maintaining the stability of LSD1.

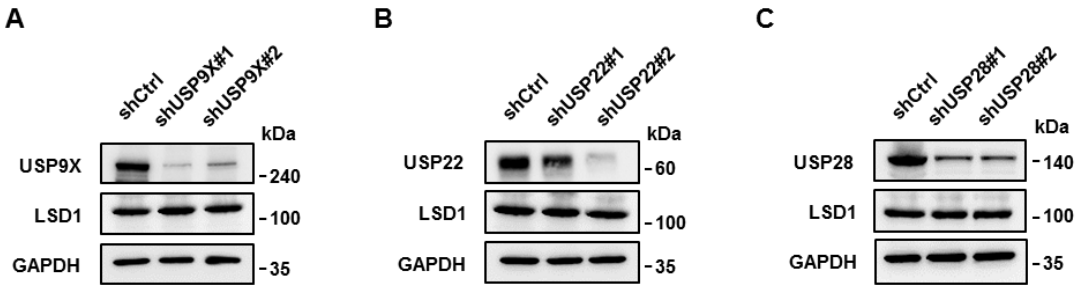


Figure R2. MDA-MB-231 cells were transfected with USP9 (A), USP22 (B) and USP28 (C) shRNAs or control vector, respectively. And the LSD1 protein level was assessed by immunoblotting.

Besides, we further examined the ubiquitination of LSD1 after knockdown of these four deubiquitylases (DUBs), and the results showed that LSD1 ubiquitination was increased after USP7 knockdown (seen new Fig EV4C), whereas the other three DUBs failed to do so (Figure R3 for referee). Collectively, these results suggest that only USP7 deubiquitinates and stabilizes LSD1.

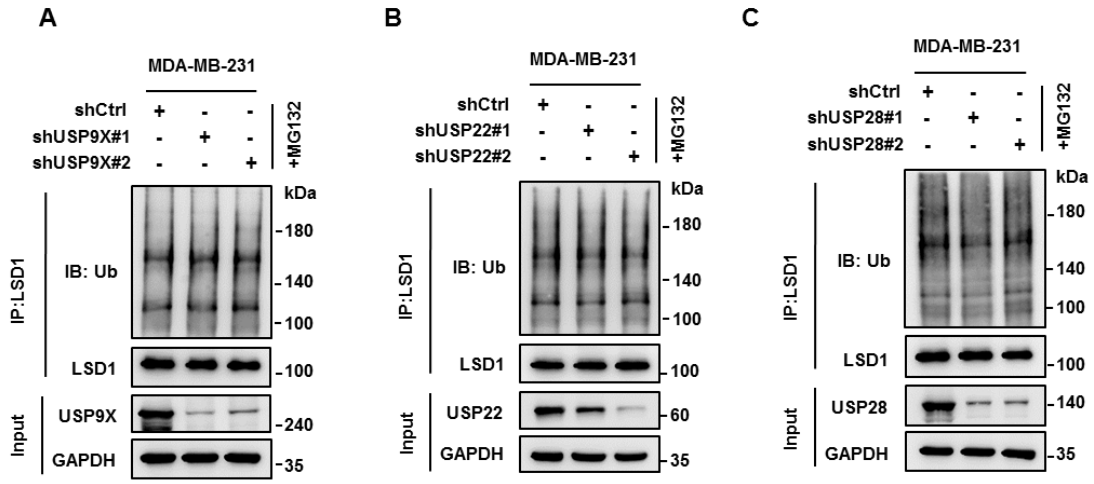
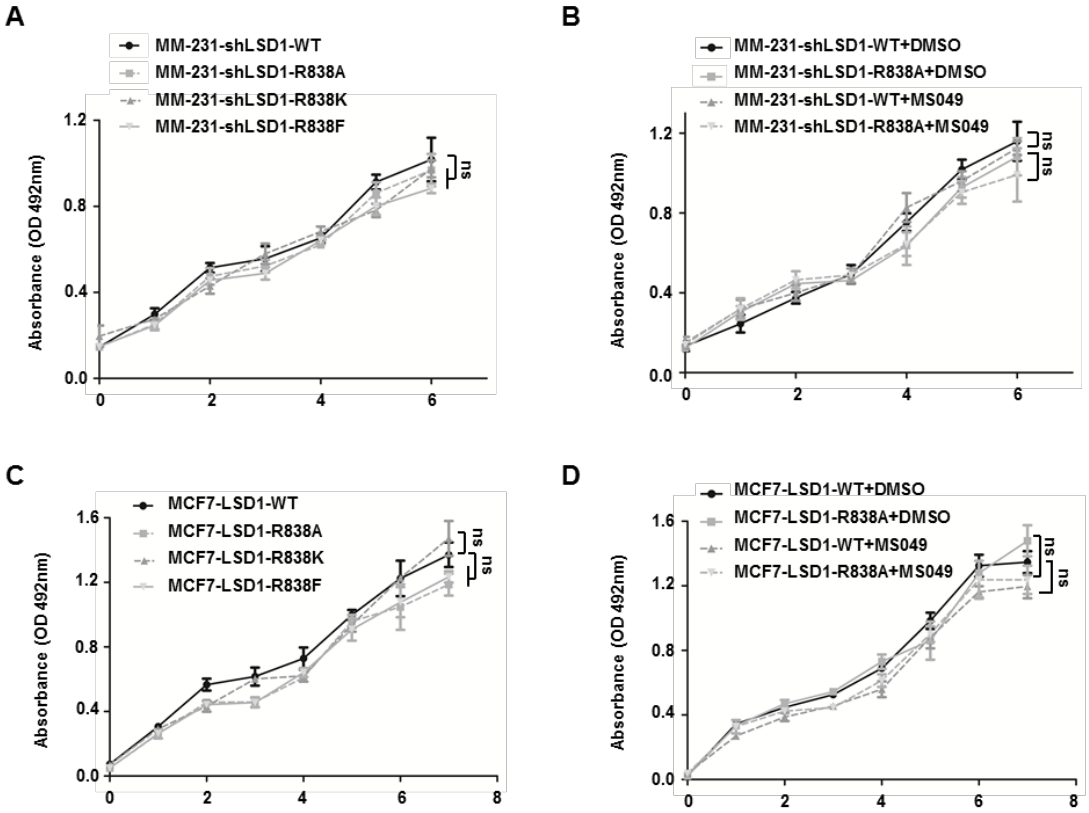


Figure R3. The ubiquitination level of endogenous LSD1 was assessed by immunoblotting in USP9- (A), USP22- (B) and USP28- knockdown (C) MDA-MB-231 cells.

As shown in Fig 3A, although all the 4 DUBs can bind to LSD1, the mutation of LSD1 on Arg838 only affects the binding of USP7, but not the other 3, indicating that LSD1 R838 methylation has no correlation with USP9X, USP28 and USP22 under our experimental system. However, the relationship between these DUBs and LSD1, and whether they affect the function of LSD1 remains to be further studied.

Q3: The authors showed results on methyl-LSD1 in migration and invasion. It is important also characterize cell proliferation.

Response: We think this is an important point. Following this suggestion, we explored the role of LSD1 R838 methylation in cell proliferation by using MTT assay. And the results showed that the growth rate of MDA-MB-231 cells expressing LSD1 WT was similar to that of LSD1 mutants (R838A/R838K/R838F) (new Appendix Fig S4A). We then treated MM-231-shLSD1-WT and MM-231-shLSD1-R838A cells with PRMT4 inhibitor MS049 and found that the growth velocity also displayed a negligible change (new Appendix Fig S4B). Similar results were also seen in MCF7 cell lines under the same treatments (new Appendix Fig S4C-D). These data suggest that methylation of LSD1 at R838 has no impact on cell proliferation. Corresponding new results have been presented in Appendix Fig S4 and Result 4 in the revised manuscript.



Appendix Fig S4. The impact of LSD1 R838 methylation on cell proliferation, and the growth rate of MM-231 and MCF7 cell lines were measured by MTT assays.

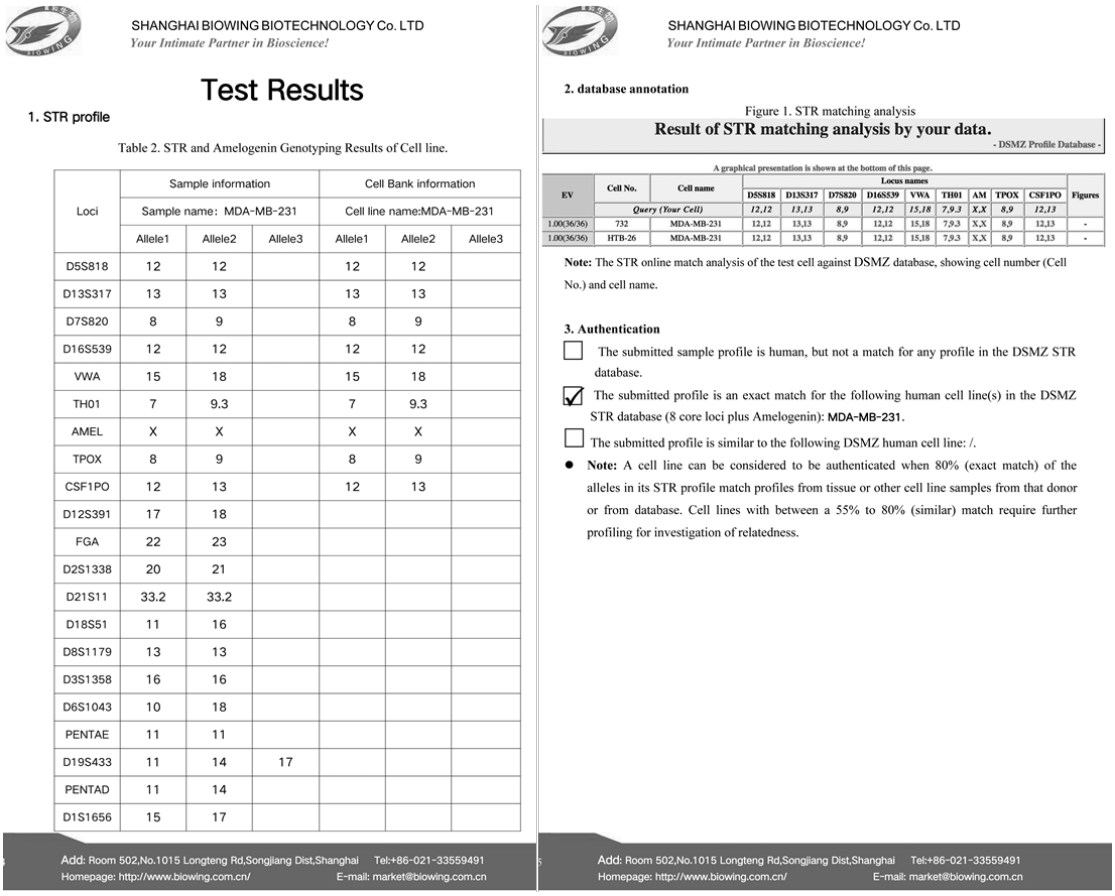
Q4: MCF710A is a non-tumorigeneic mammary epithelial cells. The use of this cell in migration assay in Figure 4C and D is not justified. MCF7 or other breast cancer cell lines should be used.

Response: We are grateful to the referee for this very constructive and helpful suggestion. We have replaced the Fig 4C and D with the results from the experiments using MCF7 cell lines, and the old Fig 4C and D are now Fig EV 5C-D in the revised manuscript. More details can be found in Results 4.

Minor comments:

Q5: Original MDA-MB-231 is in general not very metastatic, only the selected clone LM2 is metastatic. It is surprising that massive lung metastasis could be observed in Fig. 4E, F. The authors should validate this cell line by STR analysis.

Response: We agree with the referee. Actually, R838A mutant did have fewer lung metastases foci, and the massive lung metastasis, which might be triggered by overexpressed methyl-LSD1. As stated in the text, we found that LSD1 R838 methylation remarkably increased migratory and invasive capabilities of breast cancer cells (seen Figs 4A-D and EV5). MDA-MB-231 cell line used in the present manuscript was obtained from the American Type Culture Collection (ATCC, ATCC® Number: CRM-HTB-26™ ; Lot Number: 61376207). To address the referee's concern, we validate this cell line by STR analysis (Testing Company: Shanghai Biowing Applied Biotechnology Co. Ltd, China). Here we attached the main part of "STR Profiling Report" below.



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Test Results

1. STR profile

Table 2. STR and Amelogenin Genotyping Results of Cell line.

Loci	Sample information			Cell Bank information		
	Sample name: MDA-MB-231			Cell line name:MDA-MB-231		
	Allele1	Allele2	Allele3	Allele1	Allele2	Allele3
D5S818	12	12		12	12	
D13S317	13	13		13	13	
D7S820	8	9		8	9	
D16S539	12	12		12	12	
VWA	15	18		15	18	
TH01	7	9.3		7	9.3	
AMEL	X	X		X	X	
TPOX	8	9		8	9	
CSF1PO	12	13		12	13	
D12S391	17	18				
FGA	22	23				
D2S1338	20	21				
D21S11	33.2	33.2				
D18S51	11	16				
D8S1179	13	13				
D3S1358	16	16				
D6S1043	10	18				
PENTAE	11	11				
D19S433	11	14	17			
PENTAD	11	14				
D1S1656	15	17				

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2. database annotation

Figure 1. STR matching analysis

Result of STR matching analysis by your data.
- DSMZ Profile Database -

A graphical presentation is shown at the bottom of this page.

EV	Cell No.	Cell name	Locus names								Figures
			D5S818	D13S317	D7S820	D16S539	VWA	TH01	AM	TPOX	CSF1PO	
	Query (Your Cell)		*12,12*	*13,13*	*8,9*	*12,12*	*15,18*	*7,9.3*	*X,X*	*8,9*	*12,13*	
1.00(36/36)	732	MDA-MB-231	12,12	13,13	8,9	12,12	15,18	7,9.3	X,X	8,9	12,13	-
1.00(36/36)	HTB-26	MDA-MB-231	12,12	13,13	8,9	12,12	15,18	7,9.3	X,X	8,9	12,13	-

Note: The STR online match analysis of the test cell against DSMZ database, showing cell number (Cell No.) and cell name.

3. Authentication

☐ The submitted sample profile is human, but not a match for any profile in the DSMZ STR database.

☑ The submitted profile is an exact match for the following human cell line(s) in the DSMZ STR database (8 core loci plus Amelogenin): MDA-MB-231.

☐ The submitted profile is similar to the following DSMZ human cell line: /.

- **Note:** A cell line can be considered to be authenticated when 80% (exact match) of the alleles in its STR profile match profiles from tissue or other cell line samples from that donor or from database. Cell lines with between a 55% to 80% (similar) match require further profiling for investigation of relatedness.

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Q6: The different mechanism of LSD1 regulating E-cadherin and vimentin is not convincing. Fig.5 should characterize the difference of occupancy of H3K4me2 and H3K9me2 on these two gene promoters.

Response: We thank the referee for raising this important point. These results are consistent with our previous reports (Feng et al., Oncogene, 2017, see Fig 5). To date, the question of what mechanisms are actually involved in the selectivity of LSD1 in its histone modification has not been properly answered, and has been an issue of research attention. At the present time, we have to say that we are unable to answer this attractive question with precision based on our existing data. Nevertheless, we have addressed the complexity of this issue in more details in the "Discussion" in the revised manuscript.

Reference: Feng J, Li L, Zhang N, Liu J, Zhang L, Gao H, Wang G, Li Y, Zhang Y, Li X, et al. (2017) Androgen and AR contribute to breast cancer development and metastasis: an insight of mechanisms. Oncogene 36: 2775-2790.

We apologize if Fig 5 caused any confusion that made it difficult to follow. We in fact examined the occupancy of H3K4me2 and H3K9me2 on these two gene promoters. Specifically, Our ChIP results revealed that R838A mutant led to a marked increase of H3K4Me2 but unchanged H3K9me2 on E-cadherin promoter (Fig 5E); meanwhile, an increased H3K9Me2 but unchanged H3K4me2 on vimentin promoter (Fig 5F). And after treated MM-231-shLSD1-WT cells with MS049 or P5091, we found that the occupancy of H3K4me2 on E-cadherin promoter was increased notably but only a marginal change of H3K9me2 (Fig 5E); whereas, an increase of H3K9me2, but not H3K4me2 on vimentin promoter (Fig 5F). In contrast, MS049 or P5091 treatment had no influence on both promoters in MM-231-shLSD1-R838A cells. These results suggest that LSD1 methylation participates in the repression of E-cadherin and activation of vimentin genes by attenuating H3K4me2 and H3K9me2, respectively.

We have rearranged these results in the revised manuscript, and hope it will become clear now.

Referee #3:

This manuscript describes that LSD1 is methylated at R838 by arginine methyltransferase PRMT4 and this methylation enhances LSD1 protein stability. The authors found that the mutation of certain amino acid residues in the region around R838 motif reduces the binding of USP7, a deubiquitylase, which is previously shown to regulate LSD1 protein stability. While some parts of experiments are well done, there are several major problems on some critical data in the manuscript:

Q1: Methylation and methylation antibodies are not well characterized. Although R838 in LSD1 appears to be a good arginine substrate for PRMT4, as it has the consensus "L/I-P-R" methylation site conserved in other PRMT4 substrates, the prove is not very convincing. In Figure 1A, the mass spectrometry of the peptide 825-838 from of transfected LSD1 has no molecular weight indicating whether it is mono- or di-methylated, or not methylated. In Figure 1E, it seems that not only R838A but also 4RA, R608A, are reduced in the methylation assays by GST-PRMT4. R108A seemed to stimulate LSD1 methylation. There is no explanation in the text. In Figure EV2B, the test of methylation and non-methylation antibodies, how was this experiment done? How can one use the same blot to incubate it with two antibodies (Nme-ab and me-ab)? There is no description on how the me-ab is raised, purified, and blotted.

Response: We thank the referee for the questions. We apologize for the confusion caused by Fig. 1A. In fact, the mass spectrometry showed that the peptide 825-838 was di-methylated (the original data can be found in Dataset EV1), and in order to see it more clearly, we now scale it up and indicate the molecular weight at the corresponding position (as seen if Figure R4 for referee). We hope it will become clear now.

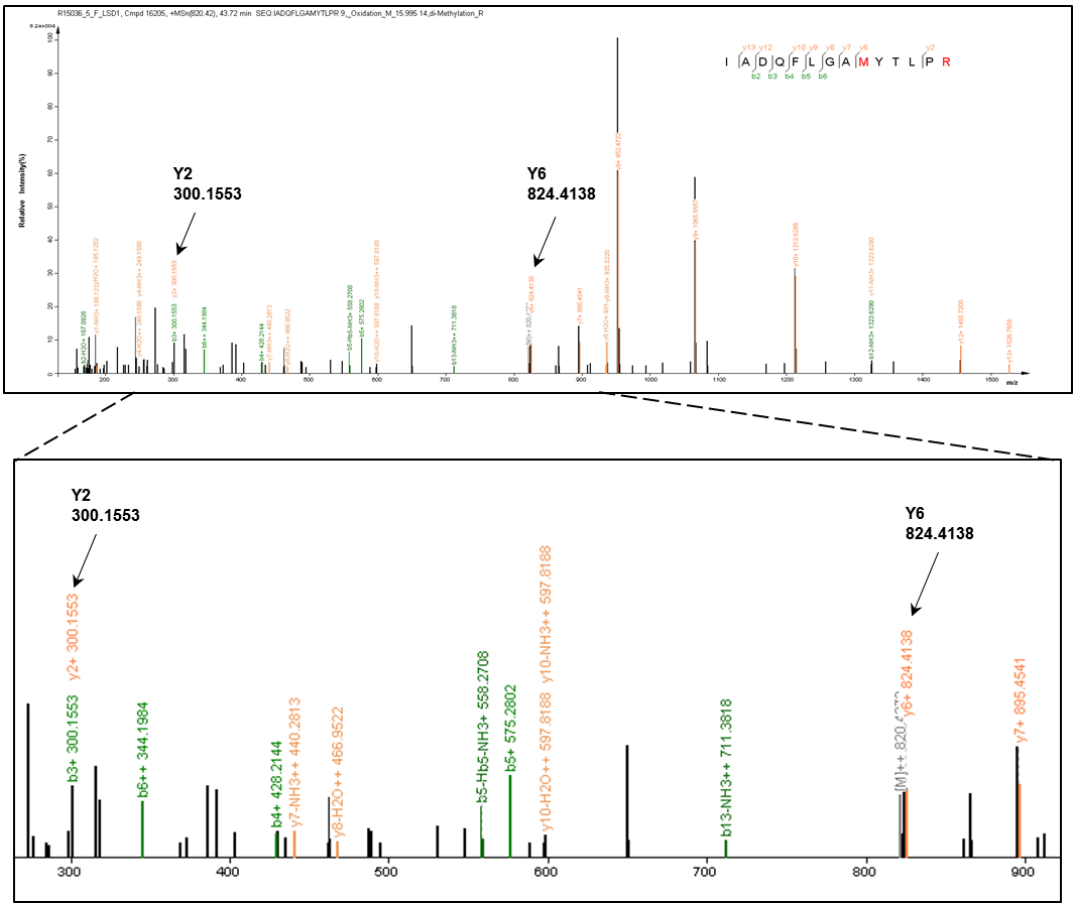

Figure R4. Mass spectrometric analysis of LSD1 (peptide 825-838).

In Figure 1E, 4RA (mutations of all 4 sites in combination) contains the mutation of R838A, so the two lanes showed similar results. As for R608A and R108A, the differences in LSD1 methylation might be caused by the difference in the amount of loading of these two bands. In fact, we repeated the experiment and replaced it with another one and we hope the new figure is satisfactory (new Fig 1E).

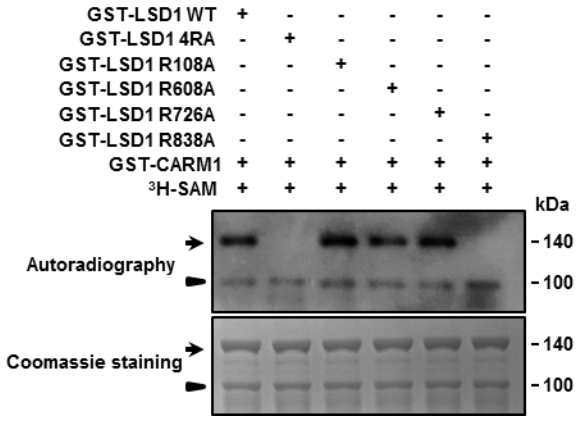

Fig 1E. *In vitro* methylation assays. Methylated proteins were detected via autoradiography.

Moreover, we also analyzed the methylation of LSD1 by immunoblotting with anti-ASYM after incubated GST-tagged PRMT4 (CARM1) with GST-tagged LSD1 WT or its mutants (R108A, R608A, R726A, R838A, 4RA) in the presence of SAM. Notably, the R838A, as well as 4RA, abolished the PRMT4-mediated methylation of LSD1 (Fig EV1F).

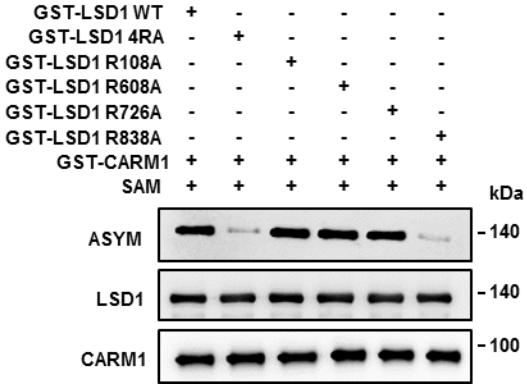

Fig EV1F. *In vitro* methylation assays. Methylation of LSD1 protein was analyzed by Western blotting.

We apologize for the confusion caused by Figure EV2B. The me-ab production was performed by GL Biochem Co., Ltd (Shanghai, China), and the dot blot data were also provided by company. Actually, the Me-peptide and Nme-peptide were spotted onto the same nitrocellulose membrane, prepare two copies and incubated with Nme-ab and me-ab, respectively. Finally, after incubation with secondary antibody, they were exposed in the same X-ray film in the dark room.

Besides, the peptide P[R-(Me)2]QATPGVPAQQSPSM, which corresponds to aa 837-852 of human LSD1 protein, was used to immunize rabbits to generate a polyclonal antibody that detected LSD1 R838 asymmetric dimethylation. The supplier company has provided us with only basic protocols, with limited details, presumably due to the business secret convention. The available information has now been added to Appendix materials and methods.

Q2: Ubiquitination assays in Figure 2H and I were not correctly conducted so it is not convincing. Since anti-LSD1 immunoprecipitation could bring down many LSD1-associated proteins that can be polyubiquitinated which can be detected by anti-Ub antibody, this data is also not convincing.
Response: It is really a good point, and we think this is complicated. As shown in Fig 2H and 2I, we performed immunoprecipitation with anti-LSD1 to assessed the ubiquitination level of LSD1, and found that the ubiquitination was sharply increased after PRMT4 knockdown or inhibition. It is true that we cannot rule out the possibility that PRMT4 may stabilize LSD1-associated proteins by reducing their ubiquitination based on our existing data. However, these results, combined with the findings in Fig 2B-E (that is: PRMT4 knockdown or inhibition can both decrease protein level of LSD1 and shorten the half-life of LSD1, while the inhibitory effect of PRMT4 knockdown or inhibition on LSD1 was reversed under proteasome inhibitor MG132 treatment), at least support the notion that PRMT4 stabilizes LSD1 by decreasing its ubiquitination.

It was worth mentioning that, the ubiquitination level of LSD1 R838A mutant was much higher than that of LSD1 WT. And more importantly, PRMT4 inhibition led to the sharp elevation of ubiquitination of LSD1 WT, whereas R838A mutant showed a negligible change (seen Fig 2L). Taken together, our data indicate that the PRMT4-dependent LSD1 R838 methylation is responsible for LSD1 stabilization.

Q3: How R838 methylation affects USP7 remains not very well characterized. The major significance of R838 by PRMT4 is that mutation of this LPR motif reduced USP7 binding in transfected cells. Previous studies (Oncology reports 36, 29935-2945, 2016) showed USP7 binds to LSD1 but did not map the binding site on LSD1. Thus, a characterization of USP7 binding to LSD1 both in vivo and in vitro would address why methylated R838 interferes with binding of USP7.

Response: We appreciate the reviewer for this constructive point. As stated in the text, we found that PRMT4-dependent methylation of LSD1 R838 is critical for LSD1 binding with USP7 that leads to the deubiquitination of LSD1.

As requested, to further dissect the interaction between LSD1 and USP7, we generated GST-tagged or Flag-tagged deletion mutants of LSD1 (Fig EV3A). The GST-tagged mutants were bacterially expressed, affinity-purified, and applied for GST pull-down assays with Flag-USP7 purified from HEK-293T cells. It was found that both the mutant with deletion of the carboxyl terminus of LSD1 (◀CT) and the mutant with deletion of the carboxyl terminal AOD (◀AOD-C) dramatically weakened the pull-down of Flag-USP7 (Fig EV3B). Furthermore, the Flag-tagged mutants were overexpressed in HEK-293T cells, followed by co-immunoprecipitation assays using anti-Flag antibody, and the same results as GST pull-down were obtained (Fig EV3C). Taken together, these results indicate that the carboxyl AOD domain of LSD1 (520-852 aas) was required for the interaction between LSD1 and USP7. These new results have been presented in Figure EV3 and Result 3.

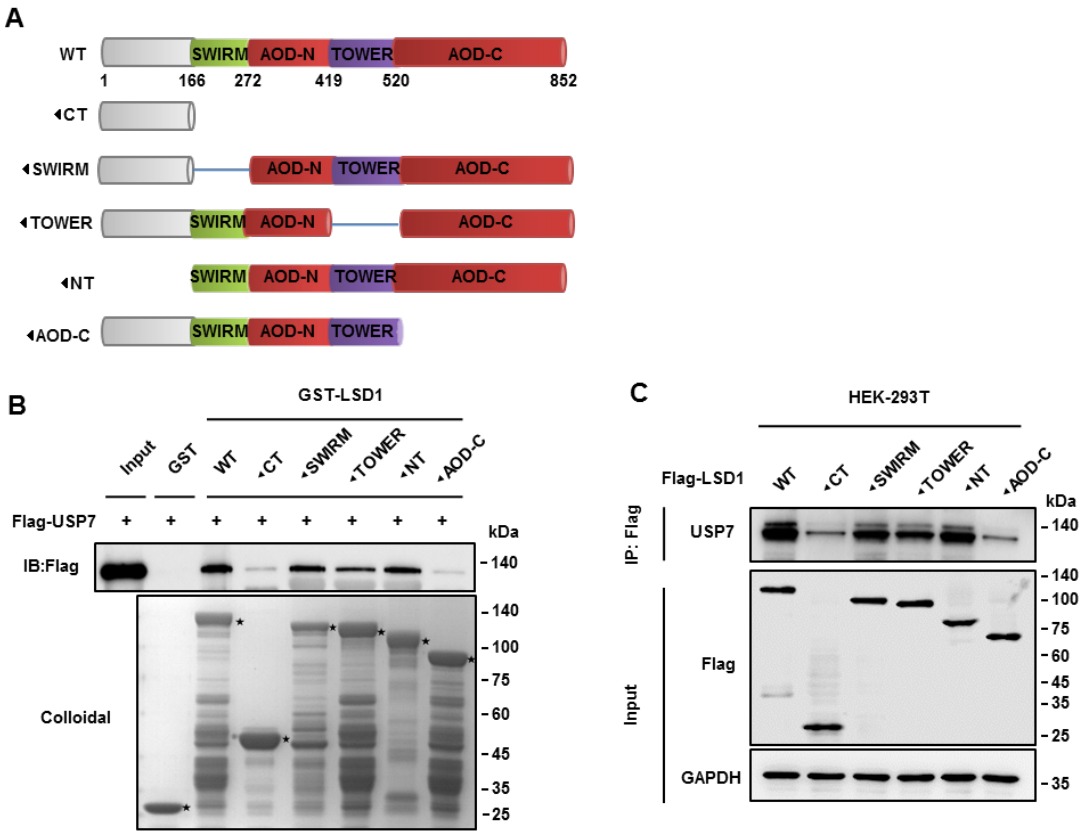

Fig EV3. Carboxyl AOD domain (AOD-C) of LSD1 is important for its interaction with USP7. GST pull-down (B) and IP (C) assays were performed to dissect the interaction between LSD1 deletion mutants and USP7.

We further tested whether R838 methylation of LSD1 affected its interaction with USP7 *in vitro*. Recombinant LSD1 was *in vitro* methylated (Figure R5A for referee) and subjected to pull-down assay with Flag-USP7 purified from HEK-293T cells. Compared with unmethylated recombinant LSD1, methylated LSD1 showed a dramatic increase in binding to USP7 (Figure R5B for referee). Together, these data support the conclusion that LSD1 R838 methylation directly enhances the binding of USP7 to LSD1.

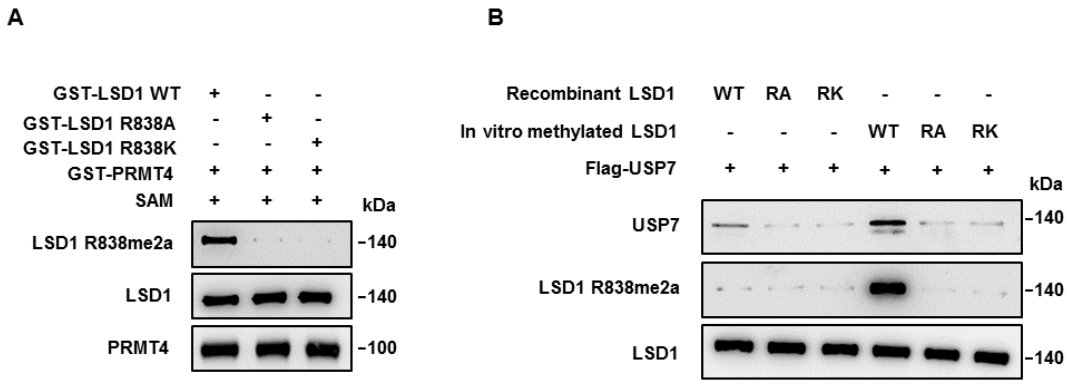

Figure R5. GST-tagged LSD1 was methylated *in vitro* (A) and subjected to pull-down assay incubated with Flag-USP7 (B).

As we known, methylation alters steric factors, hydrophobicity, and charge distribution of the guanidinium head group, and thus, protein-protein and protein-ligand interactions. However, recent studies have shown that arginine methylation modulates interactions not by a change in charge or pKa (Evich et al., Protein Science, 2016). We hypothesized that LSD1 R838 methylation results in an increase in volume of the head group and changes in steric effects, and alters the hydrophobicity, and the potential to form hydrogen bonds, all of which impact the binding of LSD1 with USP7. However, the real mechanism about how LSD1 R838 methylation affects USP7 remains to be further studied.

Reference: Evich M, Stroeva E, Zheng YG, Germann MW (2016) Effect of methylation on the side-chain pKa value of arginine. Protein Science 25: 479-486

Q4: This manuscript is also poorly written and figures are poorly labeled so it is very difficult to read. Some figures and text do not match, such as in Figure 1EV1D, the text states that "LSD1 was markedly increased after PRMT4 overexpression in HEK-293T cells". However, the figure showed LSD1 is markedly reduced. Overall, the finding is potentially interesting and may be important for the regulation of LSD1 protein levels. However, many critical experiments are not convincing.

Response: We thank the referee for these comments, and we are sorry for the confusions that arise from the improper presentation and description. Actually, in Figure 1EV1D, we performed IP assay in HEK-293T cells, and we observed that the asymmetric dimethylation status of LSD1 was markedly increased after PRMT4 (CARM1) overexpression. The lower band indicate the amount of loading indeed, and in order to avoid the confusion, we have repeated this experiment and replaced this figure by the new one (new Fig EV1D).

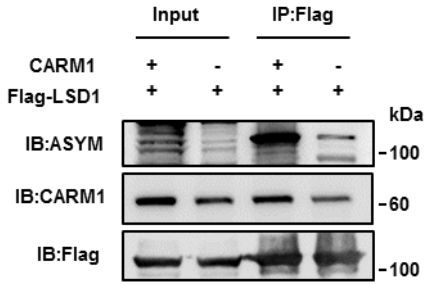

Fig EV1D. LSD1 asymmetric dimethylation status was examined by IP assay with anti-Flag in HEK-293T cells that transfected with the indicated plasmids.

We have also managed to improve and refine the English language in the text by careful proof-reading and by consulting native English speakers. We hope it now becomes more comfortable to read.

2nd Editorial Decision 7 November 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from two of the original three referees that were asked to re-evaluate your study, you will find below. Referee #1 was not able to re-assess the study, but going through your point-by-point-response, I think his/her concerns have been adequately addressed. As you will see, referee #3 has 2 remaining points to improve the manuscript I ask you to address in a final revised version. Please also provide a point-by-point-response addressing these 2 points.

Further, I have these editorial requests:

- In Fig. EV5B the 6th and 7th lower panels are identical (24h, MS049, LSD1 R838A and R838K). Please check. It seems one of the two panels is not the correct one.

- Please change the title of the part on antibody production in the Appendix file to 'Polyclonal antibody production'.

- We require that all corresponding authors supply an ORCID ID for their name upon submission of a revised manuscript. We need the ORCID of the co-corresponding author Yu Zhang. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website. This can be based on Fig. 7.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #2:

The authors have addressed my major concerns.

Referee #3:

The authors have added new figures that addressed the major concerns of my original review. However, there are some minor issues with the revised figures that may strengthen the conclusion of the manuscript:

The original and the revised Fig. 1A showed a low resolution mass spectra which are barely illegible. The authors probably should use Figure R4 (in the point-by-point response) with the scaled-up mass spectra to show the molecular weights of the methylated peptides. It would also helpful if the authors could state that these arginine residues are di-methylated according to the molecular weights of the peptides in page 5 and page 6.

Fig. EV3. The authors have showed in vitro and in vivo binding of USP7 to LSD1 is abolished by AODC deletion of LSD1, which contains R838 that is methylated by CARM1. While this new data support authors' claim that R838 methylation by CARM1 abolishes the binding of USP7. However,

to rigorously support the claim, would it be possible that the authors can directly show that the in vitro methylated LSD1 at R838 (Fig. 1 & EV1) by CARM1 cannot bind to USP7?

2nd Revision - authors' response 11 November 2019

Point-by-Point Response to referees' comments

Referee #2:

The authors have addressed my major concerns.
Response: We thank the referee for the comments.

Referee #3:

The authors have added new figures that addressed the major concerns of my original review. However, there are some minor issues with the revised figures that may strengthen the conclusion of the manuscript:
Response: We thank the referee for the comments.

-The original and the revised Fig. 1A showed a low resolution mass spectra which are barely illegible. The authors probably should use Figure R4 (in the point-by-point response) with the scaled-up mass spectra to show the molecular weights of the methylated peptides. It would also helpful if the authors could state that these arginine residues are di-methylated according to the molecular weights of the peptides in page 5 and page 6.
Response: We are sorry for the unsatisfactory quality of the image. As suggested, we now use the scaled-up mass spectra to show the molecular weights of the methylated peptides. The mass spectra analysis of LSD1 fragmentation which identified a dimethylated residue of R838 was shown in new Fig. 1A, while the other three peptides which contain R108, R608 and R726 were shown in new Appendix Fig S1 due to the limitation of layout. We hope the new figure is satisfactory (new Fig 1A and new Appendix Fig S1).

-Fig. EV3. The authors have showed in vitro and in vivo binding of USP7 to LSD1 is abolished by AODC deletion of LSD1, which contains R838 that is methylated by CARM1. While this new data support authors' claim that R838 methylation by CARM1 abolishes the binding of USP7. However, to rigorously support the claim, would it be possible that the authors can directly show that the in vitro methylated LSD1 at R838 (Fig. 1 & EV1) by CARM1 cannot bind to USP7?
Response: We thank the referee for the questions. As stated in the text, we actually found that PRMT4-dependent methylation of LSD1 at R838 enhances the binding of USP7 to LSD1. As show in Figure R5 (in the point-by-point response), GST-tagged LSD1 was methylated at R838 by CARM1 *in vitro* (Figure R5A), and then subjected to pull-down assay with Flag-tagged USP7. The methylated LSD1 showed a marked increase in binding to USP7 compared with the unmethylated one (Figure R5B, panel 1 compared to panel 4). These data directly indicate that LSD1 R838 methylation enhances the binding of USP7 to LSD1.

Accepted 18 November 2019

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Journal Submitted to: EMBO reports
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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes were based on the standard routinely used in the field. All experiments were performed more than 3 times
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes, statement included.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No formal randomization procedure was used for in vitro experiments. For in vivo studies, animals of the same age and genetic background were chosen and randomly allocated to groups.
For animal studies, include a statement about randomization even if no randomization was used.	Animals of the same age and genetic background were chosen and randomly allocated to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigator was blinded to groups.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, t test was used.

Is there an estimate of variation within each group of data?	Yes. Data are represented as mean ± SD
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The information has been shown in the "Appendix Materials and Methods" section on page 8 of Appendix
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines has been stated in "Materials and Methods" on page 20. All cell lines were tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the information has been stated in "Materials and Methods" on page 24.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, statement is included on page 24. The procedures related to animal experiments were approved by the Animal Care Committee of the Northeast Normal University, China.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance is confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	e. We provide information about the liquid chromatography-tandem mass spectrometry (LC-MS/MS) in "Materials and Methods" on page 23. The detailed LC-MS/MS data was shown in Dataset EV1 and Dataset EV2.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA