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Nicotinamide N-methyltransferase sustains a core epigenetic program that promotes metastatic colonization in breast cancer

Joana Pinto Couto, Milica Vulin, Charly Jehanno, Marie-May Coissieux, Baptiste Hamelin, Alexander Schmidt, Robert Ivanek, Atul Sethi, Konstantin Bräutigam, Anja Frei, Carolina Hager, Madhuri Manivannan, Jorge Gomez-Miragaya, Milan Obradovic, Zsuzsanna Varga, Viktor Koelzer, Kirsten D. Mertz, and Mohamed Bentires-Alj
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Bentires-Alj,

Thank you again for the submission of your manuscript (EMBOJ-2022-112559) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three reviewers for evaluation, whose comments are enclosed below.

As you will see, the referees acknowledge the potential interest and value of your findings, although they also express major concerns. In more detail, both referees #1 and #3 are concerned that the proposed role of NNMT in breast cancer metastasis is not sufficiently supported at this state (Ref#1, standfirst; ref#3, pt.3) and the specific stages and context remains unclear. Further, ref#1 states that the cell line models used to explore basal-like breast cancer properties are not representative, and defining features of the NNMT-dependent cancers remain too little characterised. Referee #3 points to discrepancies between limited H3K9 methylation changes and overall phenotype, which in his/her view questions the relevance of the novel signaling axis as compared to indirect effects of NNMT (ref#3, pts.5,6). Finally, causal involvement and details of PRMD5-COL1A1's function in the current lung metastasis remains too unclear in the experts' view (ref#1, ref#3, pt.3).

Given the overall interest stated and broader angle of your findings, we are able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline. I need to stress though that we do require strong support from the referees on a revised version of the study in order to move on to publication of the work.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below.

Please feel free to approach me any time should you have additional questions related to this.

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Best regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response 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://wol-prod-cdn.literatumonline.com/pb-

assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

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 .

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Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Jan 2023.

Referee #1:

This is a potentially interesting study on the role of NNMT and epigenetic regulation in several breast cancer cell lines. While the studies are in general carefully performed the interpretation and conclusion that the primary site of regulation is metastatic colonization is not supported by the results presented. In the two cell line models studies(see below), NNMT knockdown affects cell proliferation of primary cells and the changes reported in collagen expression and potential changes in matrix stiffness have been shown by many investigators to affect cell invasion and migration, so it is likely that intravasation is also regulated. This could have been studied in the resection study. Have the authors looks at circulating tumor cells in their studies? A second issue that is not discussed is the importance of the hybrid EMT phenotype in plasticity and metastasis. Several recent studies have reported that gene signatures corresponding to the intermediate hybrid EMT state are associated with poor patient outcome(Deshmukh et al. PNAS PMID 33941680) and this is thought to be important in metastasis. The models used in the present study, both MDA-MB231 and SUM159PT models are mesenchymal claudin-low models and are not representative of the majority of basal-like breast cancers, nor do they appear to be representative of this intermediate hybrid EMT state. Of interest is that both of these models have been reported to have a STAT3 enriched tumor initiating stem cell subpopulation(Wei Wei et al. Stem Cells, PMID 24891218). Does NNMT affect this subpopulation? MDA-MB231 cells have a ras mutation, which is not representative of most basal-like breast cancers. A derivative of the MB231 cells the LM2 derivative has increased metastatic potential, and several studies have compared the parental and LM2 derivative to investigate critical genes and pathways involved in metastasis. Does NNMT expression correlate with the increased metastatic potential of LM2?. More generally the authors should show the actual data where they looked across the large number of basal like breast cancer cell lines and PDX models at NNMT levels. The mechanistic link between collagen expression and lung colonization is unclear? The Discussion touches on this, but is there direct evidence for a role in the lung metastatic niche? As mentioned above, the analysis of NNMT and COL1A1 in breast cancers was performed in primary tumors. Again how does this relate to intravasation, dissemination,extravasation and colonization? What about its role in CAFs rather than tumor cells? Finally, no data are presented about the treatment relevance for aggressive forms of breast cancer as suggested in the Discussion? Do NNMT inhibitors affect established mets? Increasing evidence suggests early dissemination of cells even prior to the identification of palpable tumors, so it is unclear how this might be used as a therapeutic intervention. In summary a more balanced and thoughtful presentation of the data presented is required prior to publication.

Referee #2:

In the current manuscript Couto and co-authors demonstrate the role of NNMT -mediated methyl sink in supporting the expression of transcriptional factors PRMD5 and collagen genes and consequently cancer cell plasticity and metastasis. The authors demonstrate that NNMT expression is increased in metastatic lesions in comparison with the primary mammary gland/breast tumours and correlates with poor survival. Silencing NNMT expression leads to decreased metastasis and expression of collagen genes and proteins (specifically COL1A1), The authors demonstrate that inhibiting NNMT expression increases SAM/SAH ratio coinciding with increased levels of H3K9 histone methylation in general and specifically at the promoter of PRMD5 leading to the decreased expression of PRDM5. In addition they also demonstrate that NNMT KO leads to increased methylation of DNA at COL1A1 and PRMD5 promoters uncovering the mechanisms for the regulation of collagen genes and PRMD5 expression downstream of NNMT. Restored expression of COL1A1 in NNMT KOs upon increased expression of PRMD5 demonstrates the functional role of PRMD5 downstream of NNMT. The role of DNA methylation downstream of NNMT KO in partially regulating the expression of PRMD5 and collagen genes is demonstrated by using 5-aza, an inhibitor of DNA methyl-transferases. Finally, the authors demonstrate that in human tumours and cancer cell lines NNMT expression positively correlates with collagen genes, a positive correlation between NNMT and COL1A1 and PRMD5 in HR-positive breast cancer tumours.

Given that one study demonstrating the role of NNMT in promoting breast cancer metastasis by Wang et al (https://doi.org/10.1016/j.canlet.2022.215884) has only been published very recently, the current work is novel and provides strong mechanistic details for the regulation of metastatic process by NNMT through SAM and regulation of PRMD5 and collagen gene expression. I only think that some additional controls are required. Specifically:
In Figure 1f and g the authors compare metastatic burden from NNMT-KO and KO-NNMT tumours. The values from WT (control mice) should be included.

In Figure 3 adding the effect of NNMT-rescue in KO cells on SAM/SAH ratio and PRMD5 expression will further demonstrate the specificity of the effects.
In EV6 B and C PRDM5 please include the expression in WT cells.
Does PRMD5 OE in NNMT KOs restore metastasis?
Figure 4E demonstrates that 5-aza, DNA methylase inhibitor, prevents the repressive effect of NNMT KO on PRMD5 and collagen gene expression. Does 5-aza treatment also reverts NNMT-KO effect on cell invasiveness?

In Figure 5A the effect of shPRMD5 is evaluated in intravenous injection model. Why the orthotopic model (used initially to evaluate the effect of NNMT KO) has not been used? Does PRMD5 KD have any effect on the primary tumour growth?

Please reference Wang et al, Cancer Letters 2022 https://doi.org/10.1016/j.canlet.2022.215884
In the introduction accordingly.

Reference 42 is a review. Please include a more specific reference demonstrating the relationship between chromatin structure, dedifferentiation and increased mitochondrial activity.

Please double check references. For example references 87 and 88 have no relationship to SAM/SAH detection method.

Referee #3:

This paper explores the role of NNMT in breast cancer, where clinical associations reveal a correlation between NNMT and outcome. NNMT is shown to be required for metastasis of specific subtypes of cancer, with evidence that it can influence the basal state of cells, via changes in gene regulation events. Functional links with a secreted protein called COL1A1 is provided and a connection with PRDM5 is explored, with the conclusion being that changes in H3K9 methylation trigger this series of events. PRDM5 is shown to be one of the most differentially methylated genes in the presence and absence of NNMT. Follow-up functional work implicating PRDM5 in metastasis is explored and clinical correlations between the key protein players in this molecular pathway are provided.

This is a topical area of work and the majority of the data appears to be high quality. The authors have taken a comprehensive approach to explore this question and have employed sophisticated methods to identify the mechanism linking NNMT with COL1A1 and PRDM5. As is typical for this lab, the paper is well written and the figures are well presented. The link between NNMT, COL1A1 and PRDM5 is presented in a logical and linear regulatory manner, but some of the functional links and biological implications are currently missing and these issues need to be addressed.

- If NNMT is a driver of metastasis, do the levels of NNMT change in cohorts of metastases versus cohorts of primary tumor samples? Presumably existing datasets can be mined to explore this?
- The authors show that a subset of TNBC express NNMT and that these have a profoundly different outcome, but what are the other known parameters that discriminate between the NNMT positive or negative tumors? Variables such as BRCA status, Androgen Receptor levels, immune cell infiltration etc are ignored, but is it possible that known and established TNBC variables separate these cases and NNMT expression/activity is a consequence of this?
- The work on COL1A1 is limited, to the point where the findings don't add anything of substance. If COL1A1 is such a critical target, does the secretion of COL1A1 do anything of functional relevance to cancer cells or adjacent non-cancer cells?
- At the moment it is difficult to see if the changes in metastatic potential that result from NNMT or PRDM5 gene deletion/silencing is a genuine result of changes in metastasis or simply reflect changes in cellular fitness and proliferative capacity. This is particularly relevant in the in vivo models that use IV injection of cells.
- The authors suggest that there is a global change in the levels of methyl donor SAM, but the number of differential H3K9 methylation events is limited. What are the other consequences of changes in global SAM levels and why are the H3K9 methylation changes so restricted?
- The data in Figure 3d is not convincing and it's hard to see how such global changes can result is so few altered genes with differential H3K9 methylation. The lack of genes that are both differentially methylated and differentially expressed, suggests that the main conclusions might not be entirely correct. If there was a genuine global change in methylation and this then had a functional impact, how can so few genes been altered in response to methylation changes?
- Isn't PRDM5 known to be a repressor and if so, how do the authors explain its role as an activator?
- The data in Figure EV5 shows changes in oxygen consumption, suggesting that there is a movement away from glycolysis. Did the authors explore this?
- The data in Fig EV5g is not convincing. These changes are subtle and there are no numbers on how many regions are shown in the heatmap. It would be useful to know what the non-differential (i.e. a set of common regions) heatmap signal looks like between the WT and KO clones and can a couple of the key methylation changes be validated in other models?

Minor points
- The naming of the CRISPR clones is confusing and not intuitive.
- Figure 2e and 2d appear to be mixed up and does not match the reference to specific figure panels in the text.

Manuscript # EMBO Journal (EMBOJ-2022-112559R)

Point-by-point reply to the Reviewers

NNMT sustains a core epigenetic program that promotes metastatic colonization in breast cancer

Joana Pinto Couto, Milica Vulin, Charly Jehanno, Marie-May Coissieux, Baptiste Hamelin, Alexander Schmidt, Robert Ivanek, Atul Sethi, Konstantin Bräutigam, Anja L. Frei, Carolina Hager, Madhuri Manivannan, Jorge Gómez-Miragaya, Milan M.S. Obradovic, Zsuzsanna Varga, Viktor H. Koelzer, Kirsten D. Mertz and Mohamed Bentires-Alj*

We genuinely thank the Reviewers for their comments and suggestions; they have helped us to improve the quality of our manuscript. The Reviewers' comments are in blue, and our answers in black. Italic text corresponds to citation from the manuscript. The bold parts in the text refer to new figures and panels.

Referee #1:

Reviewer's comment: This is a potentially interesting study on the role of NNMT and epigenetic regulation in several breast cancer cell lines.

Author's answer: We thank the Reviewer for this comment.

Reviewer's comment: While the studies are in general carefully performed the interpretation and conclusion that the primary site of regulation is metastatic colonization is not supported by the results presented. In the two cell line models studies (see below), NNMT knockdown affects cell proliferation of primary cells and the changes reported in collagen expression and potential changes in matrix stiffness have been shown by many investigators to affect cell invasion and migration, so it is likely that intravasation is also regulated. This could have been studied in the resection study. Have the authors looks at circulating tumor cells in their studies?

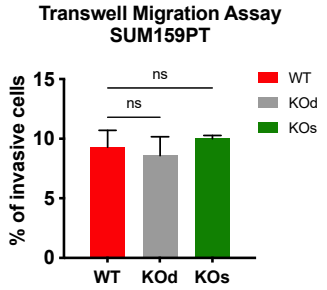
Author's answer: We thank the Reviewer for these remarks and for raising the question at which step of the metastatic cascade NNMT knockout (KO) decreases metastasis. Indeed, *in vivo*, we observe that in NNMT-KO cells, primary tumor formation is delayed as compared to NNMT-expressing cells, however once the tumors start growing, they proliferate at the same rate (Figure EV2D). Additionally, we provide data showing that there is no difference in proliferation between NNMT WT and KO cells *in vitro* (**New Figure Appendix S1c**). These data suggest that the delayed tumor growth of NNMT KO cells reflects a change in tumor initiating capacity rather than a change in proliferative capacity, which we confirmed with limiting orthotopic transplantation assays *in vivo* (Figure EV2E and **New Figure Appendix S1a**). To corroborate this result, we include additional data showing that NNMT KO cells have decreased tumor sphere forming capacities as compared to NNMT-expressing cells (**New Figure Appendix S1b**). Altogether, we conclude that NNMT loss decreases the tumor initiating capacity rather than inhibiting cell proliferation.

To address the question whether NNMT affects intravasation and circulating tumor cells (CTCs) abundance, we orthotopically injected MDA-MB-231 KO1-RFP or MDA-MB-231 KO1-NNMT cells, waited for tumors to develop and quantified the number of CTCs in the blood either by flow cytometry or by plating the blood containing the CTCs *ex vivo*. To rule out the confounding effect of tumor size, we collected the blood at different time points when there was no difference in tumor volume between NNMT-KO and NNMT-expressing cells. Both approaches revealed that there was no significant difference in CTCs numbers between NNMT-KO and NNMT-expressing cells indicating that NNMT does not impinge on the ability of cancer cells to intravasate (**New Figure Appendix S1a-f**). Additionally, we performed an *in vitro* transwell migration assay in SUM159PT WT, KOs NNMT and KOd NNMT and found no difference in transwell migration indicating that NNMT depletion does not affect migration of cells (**Figure 1 for the Reviewers**).

Finally, we provide additional evidence supporting that NNMT promotes metastatic colonization. We quantified the bioluminescence signal in the lung area at multiple early time points (6h, 12h, 18h, 24h, 48h, 72h) following tail vein injection of MDA-MB-231 KO1-RFP and KO1-NNMT cells. We found no difference between NNMT-KO and NNMT-expressing cells, suggesting that NNMT KO does not interfere with either cell survival following arrival in the lung, or with the ability of cancer to extravasate (**New Figure EV2J**).

Together, our data indicate that NNMT promotes metastatic colonization.

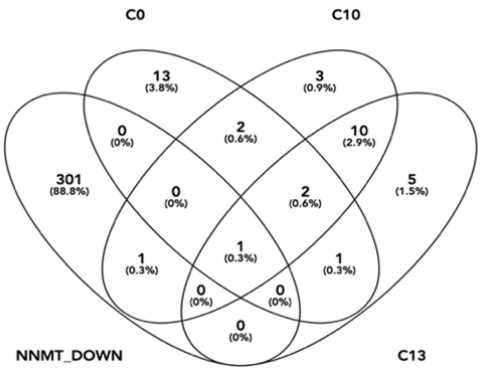
Figure 1 for the Reviewers: NNMT depletion does not affect migration in a transwell migration assay.



Reviewer's comment: A second issue that is not discussed is the importance of the hybrid EMT phenotype in plasticity and metastasis. Several recent studies have reported that gene signatures corresponding to the intermediate hybrid EMT state are associated with poor patient outcome (Deshmukh *et al*. PNAS PMID 33941680) and this is thought to be important in metastasis. The models used in the present study, both MDA-MB231 and SUM159PT models are mesenchymal claudin-low models and are not representative of the majority of basal-like breast cancers, nor do they appear to be representative of this intermediate hybrid EMT state.

Author's answer: We thank the Reviewer for raising this important point. We assessed the expression of the hybrid EMT markers in our dataset (namely CD51, CD61, CD106 and Epcam defined by Pastushenko *et al.*, 2018, Nature) and found no significant difference in expression upon NNMT knockout indicating that NNMT does not promote the hybrid EMT state. Additionally, the paper mentioned in the Reviewer's comment (Deshmukh et *al.*, 2021, PNAS PMID 33941680) shows that different signatures of hybrid EMT phenotypes are associated with poor prognosis, especially the C0, C10 and C13 signatures. An analysis in light of our data revealed almost no overlap between these gene signatures and the genes differentially down regulated upon NNMT loss suggesting that EMT hybrid phenotypes are not at play for explaining the differential metastatic ability upon NNMT loss **(Figure 2 for the Reviewers)**. What we do observe nonetheless is a loss of mesenchymal markers (Fibronectin, Vimentin) and a gain of luminal epithelial markers (Krt8/18) upon NNMT loss (Figure S4), indicating that NNMT controls tumor cell plasticity.

Figure 2 for the Reviewers: NNMT-down-regulated genes overlap with clusters C3, C10, C13 from Deshmukh et *al.*, 2021.



We thank the Reviewer for raising the question about our cellular models. We used MDA-MB-231 and SUM159PT because of the scarcity of metastatic breast cancer cell lines. Admittedly, there is no perfect model, but these cells are very commonly used in the field as a surrogate model to study metastasis (Zeng Q et *al.,* 2019, Nature - Berthelet J *et al.,* 2021, Valiente M *et al.,* 2014, Cell - Science Advance – Obradovic MMS, *et al.,* 2019, Nature - Correia AL et al., 2021, Nature – Pein M *et al.,* 2020, Nature Communications… etc). The manuscript is focused on the cellular and molecular mechanisms underlying the effect of NNMT on metastatic colonization and therefore we were limited by the currently available models. Importantly, we do validate both the clinical relevance of NNMT as a poor prognosis marker in the ERα negative subtype of breast cancer as well as the newly found NNMT-PRDM5-COL1A1 axis in clinical samples (3 PDX and > 600 primary tumor samples from breast cancer patients).

Reviewer's comment: Of interest is that both of these models have been reported to have a STAT3 enriched tumor initiating stem cell subpopulation (Wei Wei et al. Stem Cells, PMID 24891218). Does NNMT affect this subpopulation? MDA-MB231 cells have a ras mutation, which is not representative of most basal-like breast cancers.

Author's answer: We thank the Reviewer for this interesting comment which interrogates the activity of NNMT in tumor initiation and stemness. As mentioned previously, our different assays revealed that NNMT enhances the tumor initiating capacity *in vivo* (limiting dilution assays) as well as the ability to form tumorspheres *in vitro*, which thus recall stemness features (Figure EV2E and **New Figure Appendix S1a-b**). We additionally found that NNMT knockdown in the SUM159PT model decreases the $CD44^{high}$ / $CD24^{low}$ population, which delineates cancer cells in a stem-like state, thus reinforcing the effect of NNMT in promoting stemness (**New Figure Appendix S1g-h**). Besides, the functional annotation of the transcriptome of NNMT KO cells revealed several stemness related factors (such as SOX2 and KLF4) as putative regulators of the downstream target genes of NNMT,

corroborating the link with stemness (**New Figure EV3D**). Regarding the connection with STAT3, we found that the JAK/STAT3 pathway controls the expression of NNMT, as treatments of SUM159PT cells with ruxilotinib or with antibodies blocking IL6 dampened the levels of phospho Stat3 Y705 and NNMT abundance (**New Figure Appendix S1d-f**). Finally, we found that NNMT ablation does not modify the abundance of phospho Stat3 Y705. Altogether these data indicate that NNMT controls stemness properties of breast cancer cells and that its expression is dependent on the activation of the STAT3 pathway.

Reviewer's comment: A derivative of the MB231 cells the LM2 derivative has increased metastatic potential, and several studies have compared the parental and LM2 derivative to investigate critical genes and pathways involved in metastasis. Does NNMT expression correlate with the increased metastatic potential of LM2?

Author's answer: We thank the Reviewer for this interesting suggestion. To address whether NNMT could be involved in the increased metastatic potential of the LM2 cell line, we assessed the mRNA expression levels of NNMT in parental MDA-MB-231 and LM2 cell lines. We found no difference in NNMT expression in parental MDA-MB-231 and LM2 cell lines. However, at the protein level, we detected a 1.85-fold increase in the LM2 cell line as compared to the parental MDA-MB-231 (normalized to ERK2), suggesting that NNMT abundance correlates with the increased metastatic potential of LM2 (**Figure 3 for the Reviewers only**).

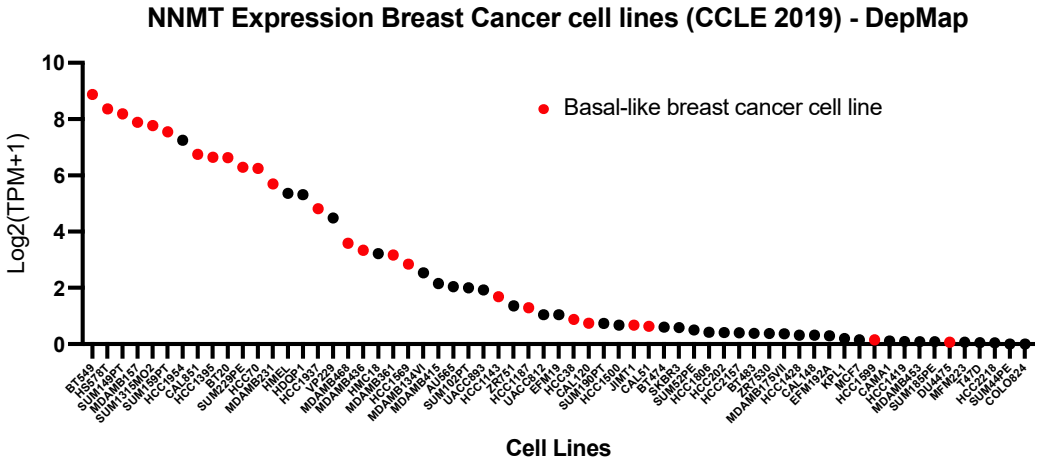
Figure 3 for the Reviewers: NNMT mRNA expression and protein abundance in MDA-MB-231 and LM2 cell lines. ERK2 was used as loading control.

Reviewer's comment: More generally the authors should show the actual data where they looked across the large number of basal like breast cancer cell lines and PDX models at NNMT levels.

Author's answer: We thank the Reviewer for this important remark. We used the CCLE resources to assess NNMT expression in multiple breast cancer cell lines belonging to the different subtypes (PAM50 classification) and found that NNMT is preferentially expressed

in basal-like breast cancer cell lines (Figure EV1 and **Figure 4 for the Reviewers only**). We then assessed NNMT expression in established PDX models from our own laboratory collection and found NNMT expressed in three of them (Figure 1). These three PDX models turned out to be all metastatic which enabled us to assess NNMT expression in primary tumors and metastasis. Interestingly, in two PDX we found elevated NNMT abundance (at the protein level) in the metastases as compared to primary tumors, while the third model showed a high level of NNMT expression regardless of the site.

Figure 4 for the Reviewers: NNMT expression across different breast cancer cell lines using the DepMap resource.
(Cell line classification according to PAM50: Marcotte *et al*., 2016, Cell)



Reviewer's comment: The mechanistic link between collagen expression and lung colonization is unclear? The Discussion touches on this, but is there direct evidence for a role in the lung metastatic niche? As mentioned above, the analysis of NNMT and COL1A1 in breast cancers was performed in primary tumors. Again how does this relate to intravasation, dissemination, extravasation and colonization?

Author's answer: We thank the Reviewer for this important comment. In the initial version of the manuscript, we relied on the literature to underline the importance of COL1A1 pre metastatic niche remodeling in the lungs, and subsequent metastatic colonization, for example:
https://www.nature.com/articles/s41586-019-0977-x#Sec21
https://www.nature.com/articles/s41556-022-00843-w
https://pubmed.ncbi.nlm.nih.gov/29906404/

These articles emphasize the effect of fibrillar collagens and collagen cross-linking enzymes in enhancing matrix stiffness and establishing the pre-metastatic niche. The originality of our findings is that collagen is derived from the cancer cells, as compared to other studies in which collagen is predominantly synthetized from the microenvironment and

specialized tissue resident cells. To address whether cancer cell-derived collagen is important for metastatic colonization in the lung, we over-expressed COL1A1 in the NNMT depleted cell lines and intravenously injected these cells to assess metastatic colonization. While NNMT-expressing cells have a higher colonizing ability as compared to NNMT depleted cells, we observed that COL1A1 over-expression in the NNMT depleted cells increases lung colonization to an intermediate level, demonstrating that cancer cell-intrinsic expression of collagen favors metastatic colonization (**New Figure 5B and new Figure EV5**).

Additionally, we demonstrate that COL1A1 knockdown in SUM159PT, alike NNMT knockdown, decreases tumor sphere forming capacities (without altering proliferation), suggesting that COL1A1 also acts on stemness properties, in a cell autonomous manner (**New Figure Appendix S8).**

We show that the NNMT/COL1A1 axis is important for initial colonization regardless of whether the axis is already active in primary samples or upregulated in metastases. NNMT-positive tumor cells secrete more collagen, which in turn enables the initial settlement of cells at distant sites. At later steps of metastatic colonization (overt metastasis), external sources of collagen come into play (e.g., from CAFs) and the analysis of COL1A1 expression in metastatic tumor cells is masked by the strong tumor cell extrinsic COL1A1 expression.

Reviewer's comment: What about its role in CAFs rather than tumor cells?

Author's answer: We thank the Reviewer for raising this important point. Here, we focused on the cancer cell-autonomous effects of NNMT. However, the effect of NNMT in CAFs has already been studied in ovarian cancer (Eckert et *al.,* 2020, Nature) as well as in a recent article working on oral squamous cell carcinoma (preprint, not peer-reviewed Shang *et al.,* https://europepmc.org/article/ppr/ppr543842).

Reviewer's comment: Finally, no data are presented about the treatment relevance for aggressive forms of breast cancer as suggested in the Discussion? Do NNMT inhibitors affect established mets? Increasing evidence suggests early dissemination of cells even prior to the identification of palpable tumors, so it is unclear how this might be used as a therapeutic intervention.

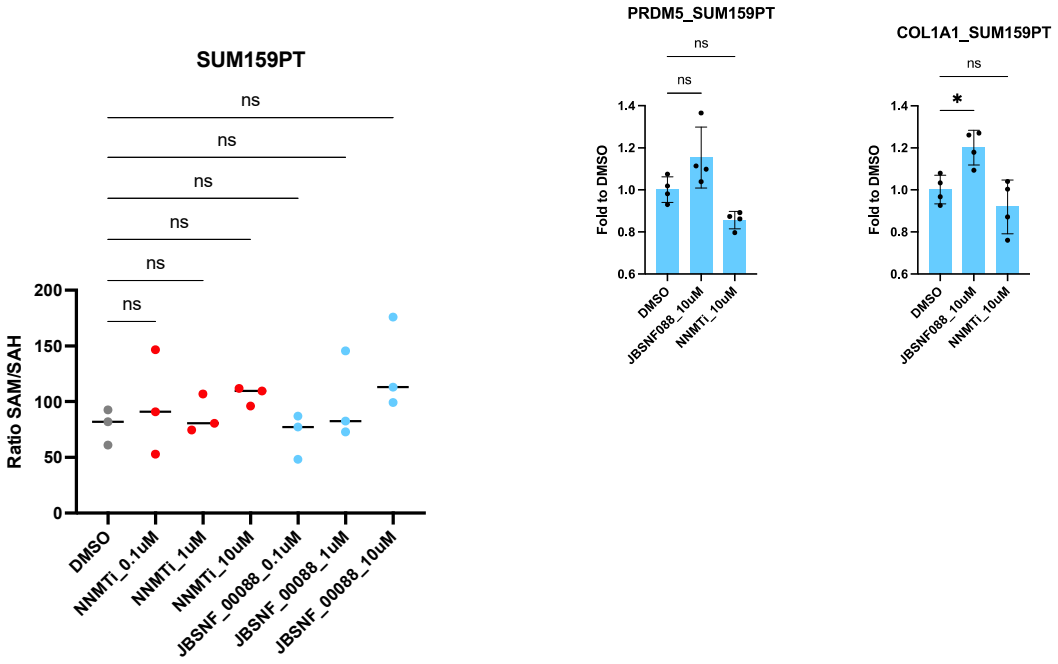
Author's answer: We thank the Reviewer for raising this important translational point. Few NNMT inhibitors do exist, such as JBSNF-000088 (6-Methoxynicotinamide) or NNMTi (5-amino-1-methylquinolinium), however little is known about their specificity, especially *in vivo*. Of note, the efficacy of JBSNF-000088 in treating metabolic disorders was assessed in pre-clinical models (Kannt *et al*., 2018, Scientific Reports). We tested the specificity of these two inhibitors by assessing their capacity to modify the SAM/SAH ratio using mass-spectrometry. Surprisingly, we found no difference in the SAM/SAH ratio between DMSO and JBSNF-000088 or NNMTi treated SUM159PT cells (**Figure 5 for the Reviewers**). Additionally, we assessed the mRNA levels of NNMT downstream targets upon NNMT

inhibitor treatments. Congruously, we found no difference in mRNA expression levels of PRDM5 and COL1A1 questioning the specificity of these inhibitors (**Figure 5 for the Reviewers**). Additional read outs can be used to assess NNMT activity such as cholesterol efflux (Wang *et al*., 2022, Cancer letters) or NAD+ abundance, however given the lack of disturbance of the SAM/SAH ratio and subsequent gene expression, we decided not to proceed further with these inhibitors for an *in vivo* metastatic assay. Besides, NNMT has been shown to be important in CAFs. The originality of our manuscript is that we discovered its cell-autonomous effects (i.e., in cancer cells). The two mechanisms are not mutually exclusive. Thus, using an inhibitor of NNMT *in vivo* will not allow us to assess its cell-autonomous effects as it will also inhibit NNMT in fibroblasts.

The development of potent and selective NNMT inhibitors capable of blocking NNMT-dependent gene expression programs is therefore warranted for attempting experiments in pre-clinical models. Given the effect of NNMT specifically on colonization and the fact that dissemination, in some situations, can occur long before primary tumor detection, the timing of administration of such inhibitors needs to be carefully established once they become available. For example, treating established metastases as proposed, or neoadjuvant or adjuvant treatment and assessing how it impinges on metastatic outgrowth could provide insights into these questions. We think that defining a relevant time-window and a stratification based on NNMT score will be required, we rephrased our discussion regarding the potential for clinical intervention (last paragraph of the discussion, please see below) to address the Reviewer's question.

"Finally, NNMT may be a potential new actionable therapeutic target for basal-like breast cancer. Indeed, few studies have described a cytotoxic effect of putative NNMT inhibitors in clear cell renal carcinoma or in melanoma, however not in the context of metastatic disease and with limited activity in vivo79,80. The development of selective NNMT inhibitors potent at increasing the SAM/SAH ratio and subsequent epigenetic programs, an accurate patient stratification based on NNMT status, and the definition of the optimal time of treatment are awaited for assessing the clinical benefit of NNMT inhibition in order to treat patients with the most aggressive forms of breast cancer."

Figure 5 for the Reviewers: Effects of JBSNF-000088 and NNMTi treatment on SAM/SAH ratio and *PRDM5* and *COL1A1* expression.



Reviewer's comment: In summary a more balanced and thoughtful presentation of the data presented is required prior to publication.

In summary, we thank the Reviewer for the comments and suggestions that significantly improved the quality of our manuscript.

Referee #2:

Reviewer's comment: In the current manuscript Couto and co-authors demonstrate the role of NNMT -mediated methyl sink in supporting the expression of transcriptional factors PRMD5 and collagen genes and consequently cancer cell plasticity and metastasis. The authors demonstrate that NNMT expression is increased in metastatic lesions in comparison with the primary mammary gland/breast tumours and correlates with poor survival. Silencing NNMT expression leads to decreased metastasis and expression of collagen genes and proteins (specifically COL1A1), The authors demonstrate that inhibiting NNMT expression increases SAM/SAH ratio coinciding with increased levels of H3K9 histone methylation in general and specifically at the promoter of PRMD5 leading to the decreased expression of PRDM5. In addition, they also demonstrate that NNMT KO leads to increased methylation of DNA at COL1A1 and PRMD5 promoters uncovering the mechanisms for the regulation of collagen genes and PRMD5 expression downstream of NNMT. Restored expression of

COL1A1 in NNMT KOs upon increased expression of PRMD5 demonstrates the functional role of PRMD5 downstream of NNMT. The role of DNA methylation downstream of NNMT KO in partially regulating the expression of PRMD5 and collagen genes is demonstrated by using 5-aza, an inhibitor of DNA methyl-transferases. Finally, the authors demonstrate that in human tumours and cancer cell lines NNMT expression positively correlates with collagen genes, a positive correlation between NNMT and COL1A1 and PRMD5 in HR-positive breast cancer tumours.

Given that one study demonstrating the role of NNMT in promoting breast cancer metastasis by Wang et al (https://doi.org/10.1016/j.canlet.2022.215884) has only been published very recently, the current work is novel and provides strong mechanistic details for the regulation of metastatic process by NNMT through SAM and regulation of PRMD5 and collagen gene expression.

Author's answer: We thank the Reviewer for the accurate summary of our findings and for highlighting the strong mechanistic aspects, and pointing to the novelty of our study.

Reviewer's comment:
I only think that some additional controls are required. Specifically:
In Figure 1f and g the authors compare metastatic burden from NNMT-KO and KO-NNMT tumours. The values from WT (control mice) should be included.

Author's answer: We thank the Reviewer for this important remark. After extensive trials in MDA-MB-231, we could only generate one clone with a complete NNMT depletion (MDA-MB-231 KO1-RFP). Since this is one clone, the proper control was the re-expression of NNMT in that same clone (MDA-MB-231 KO1-NNMT, Figure EV2). Therefore, we did not include the multiclonal parental line in the initial version.

To corroborate our findings, we provide here data from an *in vivo* metastasis assay in which we compared the MDA-MB-231 short-hairpin NT cell line with NNMT knockdown lines using 2 independent short-hairpin RNAs. As with the knockout studies, knockdown of NNMT decreased metastatic burden. We included these data in the new version (**New Appendix Figure S2a-b**).

Reviewer's comment: In Figure 3 adding the effect of NNMT-rescue in KO cells on SAM/SAH ratio and PRMD5 expression will further demonstrate the specificity of the effects.

Author's answer: We thank the Reviewer for raising this point. In order to assess whether NNMT re-expression in NNMT KO cells decreases the SAM/SAH ratio, we performed SAM and SAH quantification by mass spectrometry. Our results show that in both SUM159PT NNMT KOs and KOd, NNMT re-expression decreases the SAM/SAH ratio, thus confirming its activity in controlling SAM abundance. We included these data in the new version (**New**

Appendix Figure S4a). Additionally, we found that NNMT re-expression partially restored PRDM5 expression (mRNA level), thus confirming the specificity of our observations (**New Appendix Figure S6i**).

Reviewer's comment: In EV6 B and C PRDM5 please include the expression in WT cells.

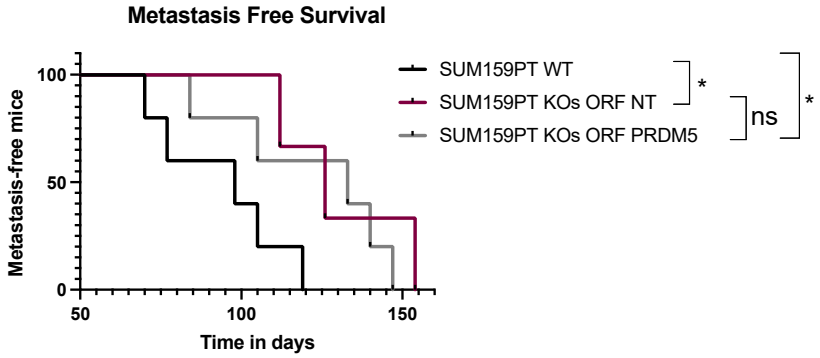
Author's answer: We included qPCR data showing PRDM5 over-expression in the WT cells, as compared to NNMT KO and PRDM5 OE lines, and thank the reviewer for this comment. Regarding figure EV6b, now figure EV4C, the over-expression of PRDM5 using CRISPR-A has already been performed in in the SUM159 WT.

Reviewer's comment: Does PRMD5 OE in NNMT KOs restore metastasis?

Author's answer: We thank the Reviewer for this very interesting question. To address this point, we engineered SUM159PT NNMT KO cell lines to overexpress PRDM5, which increased collagen gene expression (**New Figure 3G and H**).

To assess whether PRDM5 OE in NNMT KOs cells restores metastasis *in vivo,* we injected SUM159PT WT, NNMT KOs and NNMT KOs PRDM5 OE cells orthotopically, removed primary tumor and monitored metastasis outgrowth over time. We found that the overexpression of PRDM5 in NNMT KO cells did not revert the phenotype mediated by NNMT ablation (**Figure 6 for the Reviewers**). As explained in the manuscript, NNMT loss does not impair collagen gene expression only via promoter methylation of PRDM5, but also via direct DNA methylation of collagens and ECM related genes. Therefore, and not surprisingly, the sole over-expression of PRDM5 in the NNMT KO cells does not revert the metastasis phenotype due to the epigenetic barrier installed by NNMT loss.

Figure 6 for the Reviewers*:* PRDM5 OE does not rescue the metastatic phenotype of SUM159PT NNMT KO lines.

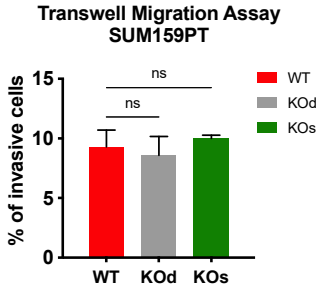


Legend: Kaplan–Meier plot depicting metastasis onset after primary tumor removal in mice injected with SUM159PT WT (n = 8), NNMT-KOs ORF NT (n = 8) and NNMT-KOs ORF PRDM5 (n = 8) cells. *$P < 0.05$; ns: not significant. Log-Rank test.

Reviewer's comment: Figure 4E demonstrates that 5-aza, DNA methylase inhibitor, prevents the repressive effect of NNMT KO on PRMD5 and collagen gene expression. Does 5-aza treatment also reverts NNMT-KO effect on cell invasiveness?

Author's answer: We thank the Reviewer for this interesting comment. In the initial version of the manuscript, we did not investigate the effects of NNMT on invasiveness. Here, we performed a transwell migration assay and found no significant effect of NNMT ablation (**Figure 7 for the Reviewers only**), therefore we did not assess the effect of 5'aza further.

Figure 7 for the Reviewers: NNMT KO does not modify migration in a transwell assay.



However, in case the Reviewer was asking whether 5-aza treatment could revert the colonization phenotype *in vivo*, it is an interesting but challenging experiment. Indeed, 5-aza treatment, despite increasing the expression of COL1A1 and PRDM5 in NNMT KO cells (Figure 4E and **New Appendix Figure S7**), displays important cytotoxic effects on cancer cells. So, it is conceivable that the benefit of restoring collagen expression by 5-aza will be hindered by the massive cytotoxic activity. Instead of treating the cells with 5-aza, we performed an *in vivo* experiment in which we restored COL1A1 expression and found that it partially reverts the ability of NNMT KO cells to colonize the lungs (**New Figure 5B**).

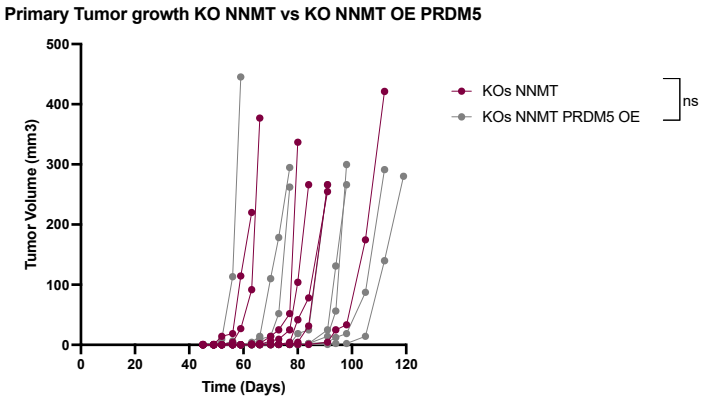
Reviewer's comment: In Figure 5A the effect of shPRMD5 is evaluated in intravenous injection model. Why the orthotopic model (used initially to evaluate the effect of NNMT KO) has not been used?

Author's answer: We thank the Reviewer for this interesting question. The orthotopic injection recapitulates the whole metastatic cascade. As we wanted to address the specific effects of PRDM5 knockdown on colonization of a distant organ specifically, we used the intravenous injection assay.

Reviewer's comment: Does PRMD5 KD have any effect on the primary tumour growth?

Author's answer: We thank the Reviewer for raising this important point. We did not assess the effect of PRDM5 knockdown on primary tumor growth. However, we did assess the effect of PRDM5 overexpression in NNMT depleted cells on primary tumor growth and found no differences (**Figure 8 for the Reviewers**), indicating that PRDM5 does not impinge on primary tumor growth *in vivo*. Additionally, we show that PRDM5 knockdown does not affect cell proliferation *in vitro* (**New Figure EV4H**).

Figure 8 for the Reviewers: PRDM5 overexpression has no effect on primary tumor growth



Legend: Graph depicting primary tumor volume over time in mice injected with SUM159PT NNMT-KOs ORF NT (n = 8) and NNMT-KOs ORF PRDM5 (n = 8) cells. ns: not significant. t-test.

Reviewer's comment:
Please reference Wang et al, Cancer Letters 2022 https://doi.org/10.1016/j.canlet.2022.215884 In the introduction accordingly.

Author's answer: We thank the Reviewer for this remark. We incorporated this important reference in the introduction and discussion sections.

Reviewer's comment: Reference 42 is a review. Please include a more specific reference demonstrating the relationship between chromatin structure, dedifferentiation and increased mitochondrial activity.

Author's answer: We thank the Reviewer for this important comment. How metabolism sustains the stem-like state is an area of intense investigation. We included this reference instead: Sperber, H. *et al*. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat. Cell Biol. 17, 1523–1535 (2015).

This article addresses why and how pluripotent cells enter the highly glycolytic cancer-like state (Warburg effect) and how it reverberates on genome hypomethylation. Indeed, stem cells tend to rely on glycolysis rather than mitochondrial oxidative

phosphorylation as glycolysis generates multiple substrates such as acetyl-CoA (https://www.nature.com/articles/nature22405), which favor genome acetylation rather than methylation, thus enabling a more permissive and plastic cell state. We rephrased the discussion to clarify this.

Reviewer's comment: Please double check references. For example, references 87 and 88 have no relationship to SAM/SAH detection method.

Author's answer: We thank the Reviewer for this important remark. We apologize for this mistake, the correct reference for SAM/SAH detection has been included in the bibliography: Sperber, H. *et al*.. Nat. Cell Biol, (2015).

Referee #3:

Reviewer's comment: This paper explores the role of NNMT in breast cancer, where clinical associations reveal a correlation between NNMT and outcome. NNMT is shown to be required for metastasis of specific subtypes of cancer, with evidence that it can influence the basal state of cells, via changes in gene regulation events. Functional links with a secreted protein called COL1A1 is provided and a connection with PRDM5 is explored, with the conclusion being that changes in H3K9 methylation trigger this series of events. PRDM5 is shown to be one of the most differentially methylated genes in the presence and absence of NNMT. Follow-up functional work implicating PRDM5 in metastasis is explored and clinical correlations between the key protein players in this molecular pathway are provided.

Author's answer: We thank the Reviewer for the clear summary of our findings.

Reviewer's comment: This is a topical area of work and the majority of the data appears to be high quality. The authors have taken a comprehensive approach to explore this question and have employed sophisticated methods to identify the mechanism linking NNMT with COL1A1 and PRDM5. As is typical for this lab, the paper is well written and the figures are well presented. The link between NNMT, COL1A1 and PRDM5 is presented in a logical and linear regulatory manner, but some of the functional links and biological implications are currently missing and these issues need to be addressed.

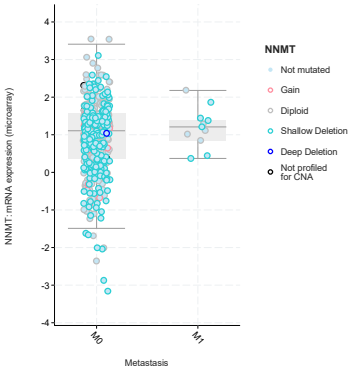
Author's answer: We thank the Reviewer for highlighting the relevance of our findings and for emphasizing the high quality of our data.

Reviewer's comment: 1) If NNMT is a driver of metastasis, do the levels of NNMT change in cohorts of metastases versus cohorts of primary tumor samples? Presumably existing datasets can be mined to explore this

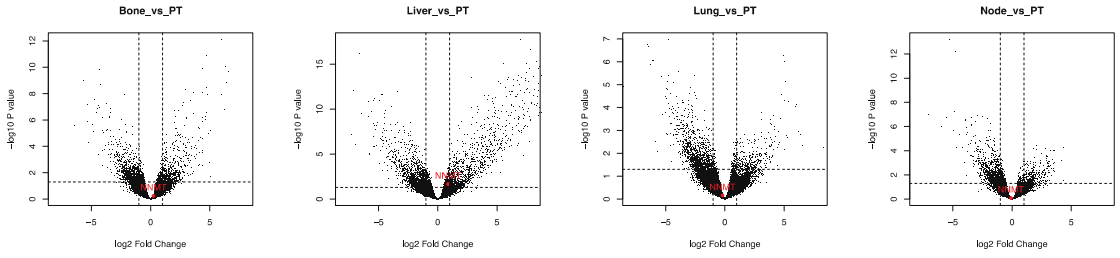
Author's answer: We thank the Reviewer for this interesting suggestion. We assessed NNMT expression in primary tumors *versus* overt metastases in three different datasets, including TCGA (lack of metastatic samples ≈ 10), PMID31627744 (encompassing 77 matched primary tumors and bone, live, lung and lymph node metastatic samples) and GSE147322 (encompassing 49 primary tumors, and bone and brain metastatic samples) and found no statistical difference in NNMT mRNA expression in primary tumors compared to overt metastases (**Figure 9 for the Reviewers**). First, we do not necessarily expect that NNMT is only expressed at the metastatic sites. We think that NNMT+/COL1A+ cells are already present in the primary tumor and have a selective advantage at colonizing the metastatic organs. Second, the functional assays we performed support NNMT relevance for the initial steps of colonization and its expression may not necessarily be maintained once the cancer cells have colonized and formed overt metastases.

Figure 9 for the Reviewers: NNMT expression in primary tumors *versus* metastasis

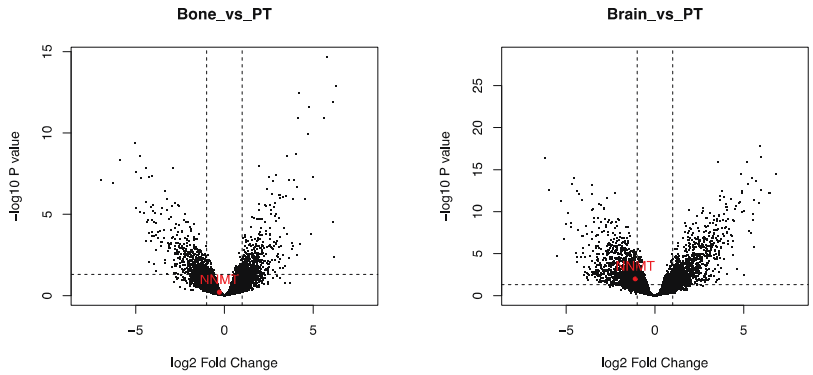
TCGA: 453 samples (but only 10 metastases)



GSE147322: 77 samples



PMID31627744: 49 samples



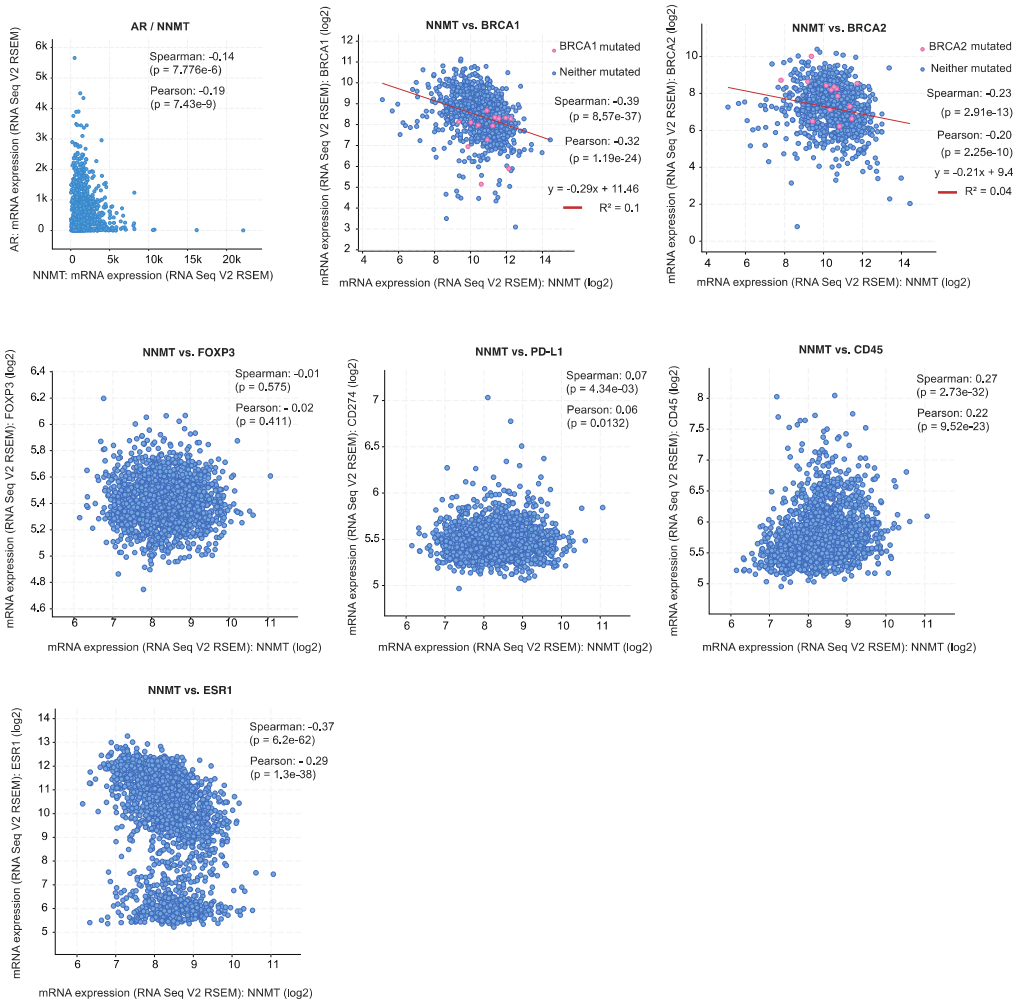
Reviewer's comment: 2) The authors show that a subset of TNBC express NNMT and that these have a profoundly different outcome, but what are the other known parameters that discriminate between the NNMT positive or negative tumors? Variables such as BRCA status, Androgen Receptor levels, immune cell infiltration etc are ignored, but is it possible that known and established TNBC variables separate these cases and NNMT expression/activity is a consequence of this?

Author's answer: We thank the Reviewer for raising this point. Indeed, from our TMA approach we correlated high levels of NNMT abundance with worst of overall survival, and that NNMT is specifically enriched in hormone-receptor negative subtypes. Unfortunately, apart from parameter such as hormone receptor status, HER2-2 status, age, post-menopause status, OS, PFS, % of tumor tissue and histotype, our TMA did not encompass further data regarding BRCA1/2 status, androgen receptor status or immune infiltration, therefore we cannot perform this correlation.

Nonetheless, we investigated publicly available datasets such as METABRIC and correlated NNMT expression with different factors (**Figure 10 for the Reviewers**). As a positive control, we included the contrast between ESR1 and NNMT1 which confirmed strong anti-correlation. Regarding immune cell infiltration, we did not detect a correlation (positive or negative) between NNMT and FOXP3 which is specifically expressed in T-lymphocytes, suggesting that NNMT status does not correlate with T cell infiltration. We observed a moderate positive correlation between NNMT and CD45 which is a pan-immune cell marker, suggesting that NNMT positive tumors might have infiltration of immune cells other than T cells. To what extent NNMT status influences the tumor immune microenvironment remains to be investigated. A recent study revealed that the reaction product of NNMT, 1-MNA, released by stromal cells, is an immune-suppressor metabolite that inhibits T cell activity in ovarian cancer (Kilgour et *al.*, 2021, Science Advances). Of note, NNMT was not found expressed in immune cells. A proper immune profiling together with relevant functional assays are therefore required to investigate this observation further. These correlations are relevant as primary tumors have been profiled by bulk RNA sequencing which therefore contain immune-related genes. Additionally, we did not find a correlation between NNMT and androgen receptor, which is rather consistent as AR is

mostly expressed in luminal tumors (and not basal-like). Interestingly, we found a negative correlation between NNMT expression and BRCA1 and BRCA2 expression (too few samples to infer any conclusion with point BRCA mutation). This observation suggests that NNMT high-expressing tumors have low levels of BRCA1/2 expression and could therefore be sensitive to targeted therapies such as Olaparib, however this is pure speculation. Importantly, these correlations could also be biased by the fact that NNMT is predominantly expressed in ERα negative, that are characterized by a higher immune cell infiltration and high prevalence of BRCA mutations. To what extent NNMT is associated with actionable therapeutic vulnerabilities is an interesting and valid question that warrants additional investigation.

Figure 10 for the Reviewers: Clinical correlation of NNMT with different factors.



Reviewer's comment: 3) The work on COL1A1 is limited, to the point where the findings don't add anything of substance. If COL1A1 is such a critical target, does the secretion of COL1A1 do anything of functional relevance to cancer cells or adjacent non-cancer cells?

Author's answer: We thank the Reviewer for this important comment. In the initial version of the manuscript, we relied on the literature to underline the importance of COL1A1 pre metastatic niche remodeling in the lungs, and subsequent metastatic colonization, for example:
https://www.nature.com/articles/s41586-019-0977-x#Sec21
https://www.nature.com/articles/s41556-022-00843-w
https://pubmed.ncbi.nlm.nih.gov/29906404/

These articles emphasize the role of fibrillar collagens and collagen cross-linking enzymes in enhancing matrix stiffness and establishing the pre-metastatic niche. The originality of our study is that we focused on collagen derived from the cancer cells, as compared to other studies in which collagen is predominantly synthetized by the microenvironment and specialized tissue resident cells. To address whether cancer cell-derived collagen is important for metastatic colonization in the lung, we overexpressed COL1A1 in the NNMT depleted cell lines and intravenously injected these cells to assess metastatic colonization. While NNMT-expressing cells have a higher colonizing ability as compared to NNMT depleted cells, we observed that COL1A1 overexpression in the NNMT depleted cells increases lung colonization to an intermediate level, demonstrating that cancer cell-intrinsic expression of collagen favors metastatic colonization (**New Figure 5B and new Figure EV5**).

Additionally, we demonstrate that COL1A1 knockdown in SUM159PT, alike NNMT knockdown, decreases tumor sphere forming capacities (without altering proliferation), suggesting that COL1A1 also acts on stemness properties, in a cell autonomous manner (**New Figure Appendix S8).**

We show that the NNMT/COL1A1 axis is important for initial colonization regardless of whether the axis is already active in primary samples or upregulated in metastases. NNMT-positive tumor cells secrete more collagen, which in turn enables the initial settlement of cells at distant sites. At later steps of metastatic colonization (overt metastasis), external sources of collagen come into play (e.g., from CAFs) and the analysis of COL1A1 expression in metastatic tumor cells is masked by the strong tumor cell extrinsic COL1A1 expression.

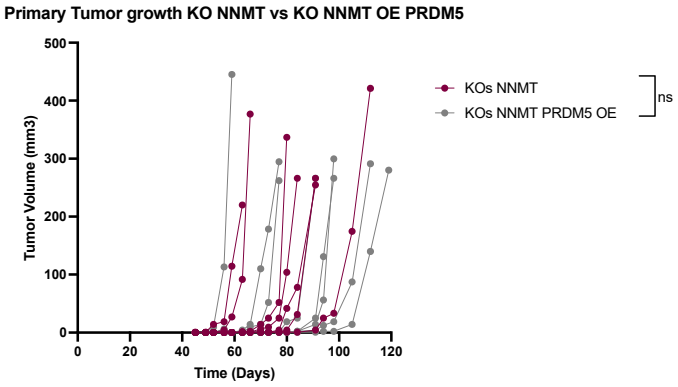
Reviewer's comment: 4) At the moment it is difficult to see if the changes in metastatic potential that result from NNMT or PRDM5 gene deletion/silencing is a genuine result of changes in metastasis or simply reflect changes in cellular fitness and proliferative capacity. This is particularly relevant in the in vivo models that use IV injection of cells.

Author's answer: We thank the Reviewer for raising this important point. To address this question, we assessed the proliferation of cells lacking either NNMT, PRDM5 or COL1A1 *in vitro* and found no significant difference (**New Figure Appendix S2, New Figure EV4H and New Figure EV5B**) indicating that NNMT, PRDM5 or COL1A1 do not directly affect proliferation.

In vivo we observe that NNMT-KO cells tumor formation is delayed compared to NNMT-expressing cells, however we observed that once the tumors start growing, they

proliferate at the same rate (Figure EV2D). The same conclusion holds true for PRDM5 (**Figure 11 for the Reviewers**). We argue instead that NNMT acts on tumor initiating capacity rather than on proliferation. Indeed, our data from limiting orthotopic transplantation assays *in vivo* demonstrate that NNMT KO have cells have decreased tumor initiating capacity as compared to NNMT- expressing, in the SUM159PT (Figure EV2E), but also in MDA-MB-231 cells, which confirms that when injected at very low numbers, only NNMT-expressing cells develop tumors (**New Figure Appendix S2a**). To corroborate this result, we include data showing that NNMT KO cells have decreased tumor sphere forming capacities as compared to their NNMT-expressing counterparts (**New Figure Appendix S2b**). Altogether, we conclude that NNMT loss decreases the tumor initiating capacity and stemness capacities rather than inhibits cell proliferation. Similarly, we found that COL1A1 knockdown decreases tumorsphere formation, thus indicating that COL1A1 also acts on stemness rather than on proliferation (**New Figure Appendix S8**).

Figure 11 for the Reviewers: PRDM5 over-expression has no effect on primary tumor growth



Legend: Graph depicting primary tumor volume over time in mice injected with SUM159PT NNMT-KOs ORF NT (n = 8) and NNMT-KOs ORF PRDM5 (n = 8) cells. ns: not significant. t-test.

Reviewer's comment: 5) The authors suggest that there is a global change in the levels of methyl donor SAM, but the number of differential H3K9 methylation events is limited. What are the other consequences of changes in global SAM levels and why are the H3K9 methylation changes so restricted?

Author's answer: We thank the Reviewer for this valid comment. Indeed, the global increase of the universal methyl donor SAM may have consequences at multiple levels other than epigenetic, such as protein or mRNA methylation (which could also explain a part of the transcriptome changes we detected), however we did not investigate further in these directions. The possibility that NNMT modifies RNA stability or protein function remains therefore open.

We rather focused on how the methyl sink effect of NNMT impinges on epigenetic, and detected elevated levels of **H3K9 methylation** (Figure 3) but also of **DNA methylation** (5mC, Figure 4), which explains at least a part of the downregulated transcriptome upon NNMT loss (how PRDM5 and collagen genes are being silenced). The question as to why the changes are centered around H3K9me3 and 5mC and not H3K27 or H3K4 is indeed a key question, and we will try to bring some explanations to it.

The methyl sink effect of NNMT has already been described in the literature and appears to be highly dependent on the cell type considered. Indeed, it has been reported that NNMT loss in cancer associated fibroblasts (CAFs) triggered changes in H3K27me3 or H3K4me3 but not in H3K9me3, me2 or me1 (Eckert et *al.,* 2020, Nature). Besides, some residues are even found to be hypomethylated despite increased SAM levels (e.g. H3K79 residue… etc). Additionally, Ulanovskaya *et al*., 2013 demonstrated that the methyl sink effect of NNMT in ovarian breast cancer cells is the most pronounced on the H3K9me3 residue and moderately on the H3K27 residue. From this observation, we can first conclude that the effect of NNMT seems to be cell type dependent.

The question of the specificity could also be explained by the enzymatic activity of the epigenetic regulators (mostly methyltransferases) associated with each mark. Indeed, their activity depends on the abundance of different metabolites such as alpha Keto Glutarate, NAD+, fumarate or succinate for which we do not have data regarding their abundance, which could explain why some epigenetic regulators are differentially sensitive to change in SAM levels. Interestingly, NNMT regulates the pool of available NAD+, which could therefore have direct consequences on the activity of the regulators that depend on it. Additionally, one can speculate that the genes downregulated upon NNMT loss can be silenced by that transcriptional repressors that recruit specific epigenetic regulators (such as DNMT or SUV39H1, associated to 5mC and H3K9me3, respectively) at the expense of other regulators associated to different epigenetic marks, thus explaining our conclusions. Of note, we did not find differentially regulated epigenetic regulators in the list of commonly regulated genes upon NNMT loss, both at the mRNA and protein level, ruling out this possibility of differential abundance. The molecular mechanisms accounting for changes restricted to H3K9me3 and DNA methylation are immensely complex, we improved the discussion in this regard, and hope these explanations answer the Reviewer's comment.

Reviewer's comment: 6) The data in Figure 3d is not convincing and it's hard to see how such global changes can result is so few altered genes with differential H3K9 methylation. The lack of genes that are both differentially methylated and differentially expressed, suggests that the main conclusions might not be entirely correct. If there was a genuine global change in methylation and this then had a functional impact, how can so few genes been altered in response to methylation changes?

Author's answer: We thank the Reviewer for this comment. The data presented in the initial manuscript were indeed showing limited effect of the H3K9me3 enrichment because we restricted our analysis to the promoter regions. This approach enabled us to identify PRDM5, which we validated as a collagen's transcriptional regulator. H3K9me3 is a repressive

epigenetic mark known for repressing repetitive elements, non-coding portions of the genome and large blocks of chromatin (heterochromatin) enabling gene silencing. We agree that the sole promoter analysis was too restricted for explaining the transcriptome. We therefore mapped H3K9me3 peaks statistically above the background noise genome-wide, and subsequently performed contrast analyses to identify H3K9me3 regions that were specifically enriched in SUM159PT NNMT KO lines as compared to WT (**New Appendix Figure S6**). Peaks to genes association using GREAT and cross analysis with the transcriptome revealed 82 genes located nearby a H3K9me3 peak. This analysis indicates that approximately one third of the downregulated transcriptome upon NNMT KO is due to H3K9me3-mediated silencing, suggesting additional processes by which NNMT influences expression of ECM components. This analysis confirmed that one of the top H3K9me3-methylated genes in NNMT KOd and KOs cells compared to WT was the transcriptional regulator PRDM5 which we also found to display significant H3K9me3 enrichment using gene bodies analysis (**New Appendix Figure S6**).

Importantly, H3K9me3 is not the only mark that we found to be elevated upon NNMT KO, but also H3K9me2 and me1, for which we did not generate ChIP-Seq data. Indeed, H3K9me2 and me1 are also described as repressive histone marks, likely to contribute to gene silencing. We also show that NNMT loss does not only increase histone methylation but also 5mC DNA methylation, which we equally validated as a key player contributing to collagen-related gene silencing (Figure 4). Altogether, we find that the accumulation of SAM reverberates on H3K9me3 (and very likely H3K9me2 and me1) but also on DNA methylation and installs an epigenetic landscape that restricts metastatic abilities of disseminated cancer cells.

Reviewer's comment: 7) Isn't PRDM5 known to be a repressor and if so, how do the authors explain its role as an activator?

Author's answer: We thank the Reviewer for this interesting question. PRDM5 comprises several Zn finger domains enabling direct DNA binding via the GAAAG motif and can serve as a platform for recruiting additional transcription factors, scaffolding proteins and/or epigenetic regulators (Hohenauer et Moore, 2012, Development: https://journals.biologists.com/dev/article/139/13/2267/45169/The-Prdm-family-expanding-roles-in-stem-cells-and). PRDM5 lacks intrinsic histone methyltransferase and has been shown both to repress transcription by recruiting G9a and HDACs enzymes for example (https://pubmed.ncbi.nlm.nih.gov/17636019/) or enhances gene expression by maintaining RNA polymerase II occupancy (Galli *et al.*, 2012, Plos Genetics: https://pubmed.ncbi.nlm.nih.gov/22589746/). The way PRDM5 functions to control gene expression remains elusive and seems to be highly context dependent, as it can both favor or repress gene expression depending on the balance of cofactors. In our cases PRDM5 favors collagen transcription, however the precise molecular mechanisms are not known.

Reviewer's comment: 8) The data in Figure EV5 shows changes in oxygen consumption, suggesting that there is a movement away from glycolysis. Did the authors explore this?

Author's answer: We thank the Reviewer for this interesting suggestion. Yes indeed, we show that NNMT KO cells have increased oxygen consumption together with increased ATP production and mitochondrial content, which argues for a switch from glycolysis to oxidative phosphorylation upon NNMT loss (Figure Appendix S5). This effect is part of the biology of NNMT, as it utilizes S-Adenosyl Methionine to convert nicotinamide into 1-methylnicotinamide. The loss of NNMT therefore increases the pool of SAM (which reverberates on chromatin methylation as our data revealed) and presumably the pool of nicotinamide NA (we did not assess it further) which can enter the NAD+ salvage cycle. The generation of NAD+ out of the excess of nicotinamide thus favoring oxidative phosphorylation and oxygen consumption (Figure Appendix S5). We did not perform additional experiments and go further into this direction.

Reviewer's comment: 9) The data in Fig EV5g is not convincing. These changes are subtle and there are no numbers on how many regions are shown in the heatmap. It would be useful to know what the non-differential (i.e. a set of common regions) heatmap signal looks like between the WT and KO clones and can a couple of the key methylation changes be validated in other models?

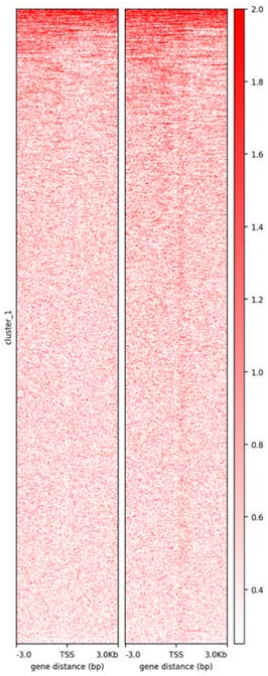
Author's answer: We thank the Reviewer for this remark. As explained in a previous answer, the analysis presented on former figure EV5g was performed on a strict selection using the H3K9me3 signal in promoter of the 301 genes found down regulated at the mRNA level.

H3K9me3 is not a mark typically known for being enriched in the promoter region, but rather in large heterochromatic blocks that contribute to chromatin locking. We therefore included additional analyses and looked for H3K9me3 enrichment in NNMT KO cells, 1) in the promoter region, 2) in the gene bodies and 3) genome wide. We found PRDM5 to be a top H3K9 trimethylated gene upon NNMT depletion (**New Appendix Figure S6**), together with a few collagen genes such as COL4A1 or COL4A2. On the new presented heat maps, we included the number of regions as well as the cut-off used.

Finally, we provide a heatmap depicting non-differential regions from the ChIP Seq analysis inferred from genes whose expressions were not altered in the transcriptome. This heatmap shows the specificity of the H3K9me3 enrichment (**Figure 12 for the Reviewers**).

Figure 12 for the Reviewers: Heatmap depicting non-differential regions from the ChIP Seq analysis inferred from genes whose expression were not altered in the transcriptome. (2000 regions).

WT NNMT KO



Reviewer's comment:

Minor points

1) The naming of the CRISPR clones is confusing and not intuitive.

Author's answer: We thank the Reviewer for pointing this out. For the SUM159PT cells, we have clarified this point in the beginning of the result section to facilitate understanding, as followed:

"*NNMT* was knocked-out (KO) in SUM159PT using two different strategies generating two independent oligoclonal KO lines, one line using a single gRNAs (referred as KOs) and another line using two gRNAs enabling genomic deletion (referred as KOd) (see Methods, Fig EV2A and EV2B)."

For MDA-MB-231 clones, we provide here more detailed explanations:
For MDA-MB-231, we could only generate one clone with a complete NNMT KO. Since this is a one cell derived clone, the proper control to include was the rescue of NNMT expression in that same subpopulation. In summary, the cell line KO1-NNMT, refers to as MDA-MB-231 cell line KO for NNMT in which NNMT has been ectopically re-expressed to the level of the parental line, while the KO1-RFP refers to as MDA-MB-231 cell line KO for NNMT in which an empty control RFP vector had been ectopically re -expressed.

2) Figure 2e and 2d appear to be mixed up and does not match the reference to specific figure panels in the text.

Author's answer: We thank the Reviewer for pointing this out and we corrected it.

Dear Dr Bentires-Alj,

Thank you for submitting your revised manuscript (EMBOJ-2022-112559-T-R) to The EMBO Journal, as well as for your patience with our response at this time of the year. Your amended study was sent back to the three referees for their re-evaluation, and we have received comments from two of them, which I enclose below. Please note that while reviewer #2 was at this time not able to give additional input, we have analyzed your response to his/her concerns editorially and found that the raised issues were satisfactorily addressed. As you will see, the other experts stated that the work has been substantially improved by the revisions and they are now broadly supportive. However, referee #3 has a remaining major technical issue with the H3K9me3 ChIP Seq analysis as illustrated in your point-by point response, which needs to be resolved.

I thus conclude that we are open to swiftly proceed towards acceptance and publication of your work, pending above matter is settled. I would appreciate if you could contact me on this point during the next weeks.

We also need you to take care of a number of minor issues related to formatting and data annotation as detailed below, which should be addressed at re-submission.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Best regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Please add up to five keywords to your manuscript.

>> Adjust the title of the 'Competing Interests' section to 'Disclosure and Competing Interests Statement'.

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.

>> Figure callouts: callout for EV2F should be after EV2E; callout for EV4F should not be before EV4A; callouts need to be added for: EV4G-H, EV5A, EV5K; callouts for EV6 should be removed, as there are 5 EV figures.

>> Author Checklist: please complete the author checklist by adding information regarding 'Experimental study design and statistics'.

>> Appendix: please add page numbers o the first page ToC.

>> Data display: please adjust data representation in Fig EV4B: n=2, to individual data points.

>> 'Data availability' section: Add a hyperlink to the GEO and PRIDE database entries and make sure to release data privacy.

>> Dataset EV legends: legends should be removed from manuscript file, and uploaded as separate tabs in each Excel file.

>> Manuscript order: place the 'Correspondence' information right after the title. Move the 'Data availability' section between 'Statistical Analysis' and 'Acknowledgments' paragraphs.

>> Remove the statement 'This manuscript contains 5 Figures, 5 Expanded View Figures, 8 Appendix Figures and 3 Datasets EV.
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Referee #1:

The authors have addressed most of my concerns with the provision of additional data, and I think the manuscript is acceptable for publication.

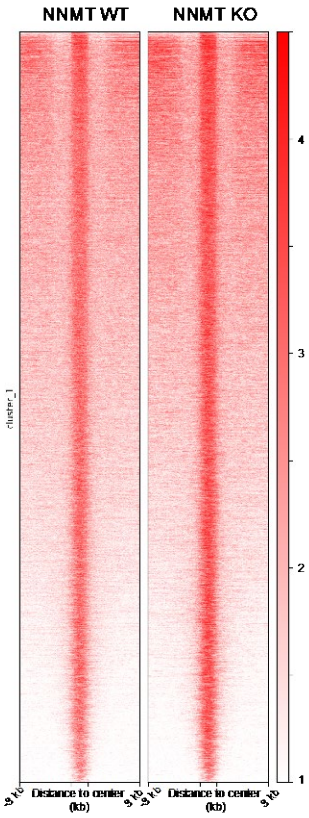
Referee #3:

The authors have taken a comprehensive approach to the reviewers comments and the new data is compelling. The new data on COL1A1 is very good and I am now convinced that this part of the work should be included in the paper. Similarly the new analysis linking peaks to genes is solid.

What I am concerned about is the ChIP-seq data. The inclusion of 'Figure 12 for reviewers' suggests that the ChIP-seq didn't work. This heatmap is not convincing and the data suggests that the binding is weak (at best) and non-existent at worst. These are supposed to represent non-differential regions and was requested so that the reader could get confidence about the quality of the overall signal from common, high confidence peaks and unfortunately it suggests that this data is not solid. The rest of the paper is high quality and I am supportive of this paper overall, but this ChIP-seq data is not convincing and it undermines the rest of the paper. In particular, the ChIP-seq in the WT conditions didn't work. Can the authors either re-analyze their data or add extra replicates?

As positive as I am about the paper, the issues with the ChIP-seq data preclude its publication.

New Figure 12 for the Reviewers: Heatmap depicting non-differential regions from the H3K9me3 ChIP Seq analysis between SUM159PT WT and NNMT KO samples. (16 000 regions).



2nd Revision - Editorial Decision **5th May 2023**

Dear Dr. Mohamed Bentires-Alj,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns by referee #3 have been well addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Best regards,

Daniel Klimmeck

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Referee #3:

The authors have addressed my concerns and the new heatmap looks good and shows solid ChIP-seq data at common regions. I am happy with the revised version of the manuscript.

EMBO Press Author Checklist

Corresponding Author Name: Mohamed Bentires-Alj	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: EMBO Journal	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOJ-2022-112559R	EMBO Reports - Author Guidelines
	Molecular Systems Biology - Author Guidelines
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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods

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Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Material and Methods

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Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Methods : Female NSG (8 to 12 weeks old) were maintained in the Friedrich Miescher Institute for Biomedical Research and in the

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Materials and Methods (including relevant references)

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgment section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	**Yes**	Material and Methods

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	For in vivo experiments (orthotopic and tail vein assays), 4 to 8 mice per group, which is sufficient to reach statisical power given the amplitude of the expected differences. We also applied the 3R principle to minimize the
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	**Yes**	Included in Materials and Methods. Randomization was performed based on age and body weight.
Include a statement about **blinding** even if no blinding was done.	**Yes**	No blinding was done.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	**Yes**	No sample was excluded from analysis
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Statistical analyses section available in Material and Methods. When data were following gaussian distribution, parametric tests were used, if not, non-parametric tests were used. Variance was similar between groups statistically compared.

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure Legends

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	**Yes**	The construction of the tissue micro arrays stained for NNMT, PRDM5 and COL1A1, was carried out under the permission of the Ethical Committee of Zurich (KEK-12-2005 amd KEK 2012-553).
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	**Yes**	Materials and methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	**Yes**	Materials and Methods and Figure legends
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.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	**Yes**	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	**Yes**	Materials and Methods
If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	Reference (#46)