

In Vivo CRISPR Screening Identifies Metastasis Suppressors in Triple-Negative Breast Cancer

Corresponding Author: Dr Jean-Jacques Lebrun

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors utilize a standard whole genome CRISPR screening library in SUM159PT cells with the goal of identifying suppressors of metastasis in an unbiased fashion. They characterize the four top hits from the screen through a variety of in vitro and in vivo assays and compellingly demonstrate that loss of any of these four genes increases metastasis and, conversely, experimental increase in expression of these genes (via CRISPR mediated promoter changes) decreases metastasis. Their in vivo analysis reveals increased CTCs across all four deleted genes and strong enhancement of disorganized vascularization in the CMTR2KO tumors. The changes in vasculature were impressively demonstrated at structural and functional levels. In vitro analysis showed increased scratch closure in 3 of 4 gene knockouts and increased Matrigel invasion in all 4. Gene expression changes were consistent with an EMT. They also analyzed public data for expression of these genes and their correlation with outcomes. The Kaplan Meier analysis showed almost overlapping trends for 3 of the 4 genes; more precision discussion of these data is essentially my only major objection. I note that the authors were scholarly in their discussion of past work and that the manuscript is well written. They also made use of multiple, complementary techniques to support most conclusions, which was quite convincing. The approach and the identification of these specific genes are likely to be broadly influential in the metastasis research field and I am highly supportive of publication of a suitably revised manuscript.

Significant:

1. The correlation of expression of each of the four genes with RFS and OS was modest for all but RBSN (which looked good). I think it is great this analysis was done and that the authors report it. However, the accompanying text is overstating the degree to which these individual genes correlate with or could be used to predict prognosis. Please edit the Results on Page 7 and Discussion on Page 11, in addition to the Abstract.

Minor:

1. Supplemental Table 1 reports the genes whose guides were overrepresented in the screen (likely suppressors). It would also be good to report the genes whose guides were underrepresented in the screen (likely enhancers) in a similar Table (or same table) to enable readers to build on this study.
2. Why was the normalization strategy changed for 6D relative to that used in 2C, I?
3. What does 5F look like without normalization for tumor size? I understand why they might normalize in that way but some small primary tumors are highly metastatic and some large tumors minimally metastatic, in patients and in experimental models.
4. BRCA is not a good abbreviation for breast cancer as it is also the abbreviation for one of the main tumor suppressors in breast cancer (e.g. title of Figure 3).

Reviewer #2

(Remarks to the Author)

Galal et al. utilize an unbiased genome-wide in vivo CRISPR/Cas9 screen in a triple-negative breast cancer (TNBC) xenograft model to identify novel metastasis suppressor genes (MSGs). The study identifies and validates VPS45, CMTR2, RBSN, and NF2 as potent suppressors, demonstrating that their ablation promotes epithelial-to-mesenchymal transition (EMT), invasion, and lung colonization, while their endogenous activation significantly abrogates metastatic potential.

Notably, the authors highlight a specific role for CMTR2 in maintaining vascular integrity, showing that its loss drives pathological angiogenesis and intra-tumoral heterogeneity that facilitate circulating tumor cell intravasation. These functional findings are corroborated by clinical analyses associating reduced expression of these targets with poor patient prognosis, suggesting they represent a network of clinically relevant barriers to TNBC progression. Here are some concerns to be addressed before publication:

Major concerns:

1. A key limitation of the immunocompromised NSG mouse model is the absence of immune cell populations. This differs significantly from human cancers, where immunosuppressive cells such as MDSCs can promote metastasis. To address this, the authors should validate the top hits in an immunocompetent mouse model, such as C57BL/6 with the E0771 cancer cell line.
2. Based on the screening design, the observed increase in lung tumor nodules could result from a combination of cancer cell seeding, proliferation, and migration. To clarify the underlying mechanism, the authors should quantify the proliferation rate for each CRISPR knockout line.
3. It would be interesting to explore potential interactions among these four genes. For example, does simultaneous knockout of two of the four genes produce a synergistic effect on tumor metastasis? Such an analysis could provide valuable insight into critical gene–gene interactions and whether they are on the same pathway or not.

Minor concerns:

1. The Surveyor Nuclease Assay shown in Figure 2A lacks professionalism. The authors should invert the image colors, as the current black background with white bands makes interpretation difficult. In addition, the authors should quantify the editing efficiency. Next-generation sequencing (NGS) is considered the gold standard for assessing gene-editing outcomes; therefore, targeted sequencing should be performed to accurately determine the INDEL frequency.
2. In the western blot image presented in Figure 2A, the knockout of VPS45 is not clearly demonstrated. Additionally, the identity of the more prominent band at the top is unclear and should be explained.
3. In the discussion section, the authors should elaborate on the therapeutic implications of identifying these four gene targets. For instance, how might one selectively activate or restore the expression of these genes in TNBC?

Reviewer #3

(Remarks to the Author)

The manuscript titled Genome-wide in vivo CRISPR screen identifies CMTR2 as a potent suppressor of metastasis in Triple-negative breast cancer presents data and evidence on the role of four tumor suppressor genes (TSGs) (VPS45, RBSN, CMTR2, and NF2) in TNBC tumor growth, lung metastasis, EMT, and angiogenesis. Notably, the study highlights CMTR2 as a key modulator of TNBC metastasis among these TSGs. The work is well-designed and offers novel insights into the functions of these genes. However, there are certain limitations in fully validating the findings based on the data provided in the current version of the manuscript.

1. The primary limitation of this study is that all cell culture experiments were conducted using a single TNBC cell line, SUM159. Findings based on one cell line are generally insufficient for publication because they lack reproducibility and generalizability. Incorporating multiple cell lines is crucial to confirm that the observed effects are not cell line specific and to strengthen the validity and broader applicability of the results. Therefore, it is essential to perform key experiments using at least one additional TNBC cell line."
2. In Figure 2H, the primary tumor size appears larger in the NF2 KO group, whereas lung metastasis is higher in the CMTR2 KO group. This observation aligns with Figure 3B, which shows a significant decrease in NF2 expression in TNBC compared to the normal group. Please discuss this point and provide a possible explanation for this finding
3. The authors report a correlation between the four TSGs and patients' relapse-free survival and overall survival (Figure 3D). It would be valuable to confirm these findings in an animal model.
4. It is important to present the expression levels of key growth factors, such as VEGF and FGF, to substantiate the claim that TSGs regulate angiogenesis in the cell culture study.
5. Were any observations made regarding the effect of these TSGs on brain metastasis?
6. Statistical Analysis: The authors used two-tailed unpaired t-tests. However, some experiments compared multiple samples to a control (Scrambled). For comparisons involving three or more group means, ANOVA (Analysis of Variance) is more appropriate than performing multiple t-tests.

Minor comments

Multiple places TNBC is written as TBNC. Please correct

Most of the results highlight the roles of four TSGs, while only a few experiments specifically examine the effect of CMTR2 alone. It would be more appropriate to revise the title to reflect the involvement of all four TSGs.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors were exceptionally responsive to the issues raised by all three reviewers, including completion of extensive new experiments. All of my concerns are satisfied. I am highly supportive of publication. I congratulate the authors on a rigorously conducted and fascinating study.

Reviewer #2

(Remarks to the Author)

The authors have responded thoroughly to my comments and have significantly strengthened the manuscript. I therefore support its publication.

Reviewer #3

(Remarks to the Author)

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors utilize a standard whole genome CRISPR screening library in SUM159PT cells with the goal of identifying suppressors of metastasis in an unbiased fashion. They characterize the four top hits from the screen through a variety of in vitro and in vivo assays and compellingly demonstrate that loss of any of these four genes increases metastasis and, conversely, experimental increase in expression of these genes (via CRISPR mediated promoter changes) decreases metastasis. Their in vivo analysis reveals increased CTCs across all four deleted genes and strong enhancement of disorganized vascularization in the CMTR2KO tumors. The changes in vasculature were impressively demonstrated at structural and functional levels. In vitro analysis showed increased scratch closure in 3 of 4 gene knockouts and increased Matrigel invasion in all 4. Gene expression changes were consistent with an EMT. They also analyzed public data for expression of these genes and their correlation with outcomes. The Kaplan Meier analysis showed almost overlapping trends for 3 of the 4 genes; more precision discussion of these data is essentially my only major objection. I note that the authors were scholarly in their discussion of past work and that the manuscript is well written. They also made use of multiple, complementary techniques to support most conclusions, which was quite convincing. The approach and the identification of these specific genes are likely to be broadly influential in the metastasis research field and I am highly supportive of publication of a suitably revised manuscript.

We sincerely thank the reviewer for the positive evaluation of our manuscript and for the constructive and insightful comments. We appreciate the recognition of the rigor of our CRISPR-based approach and the strength of our complementary in vitro and in vivo validations, including the vascular and EMT-related phenotypes. Regarding the Kaplan–Meier analyses, we agree that a more cautious interpretation is appropriate. We have revised the manuscript to refine the language, avoid overinterpretation of overlapping trends, and clearly emphasize that these data reflect associations rather than causation (see detailed answer to Rev1, point #1 (below).

We thank the reviewer again for the valuable feedback, which has helped improve the clarity and rigor of the manuscript.

Significant:

1. The correlation of expression of each of the four genes with RFS and OS was modest for all but RBSN (which looked good). I think it is great this analysis was done and that the authors report it. However, the accompanying text is overstating the degree to which these individual genes correlate with or could be used to predict prognosis. Please edit the Results on Page 7 and Discussion on Page 11, in addition to the Abstract.

We thank the reviewer for this important and insightful comment and fully agree with the concern. Upon careful re-evaluation, we confirm that among the four genes, RBSN shows the most robust and consistent association with both relapse-free survival (RFS) and overall survival (OS), whereas the remaining genes display more modest associations, generally reflecting trends toward improved outcomes with higher expression. In response, we have revised the Abstract, Results (Page 9), and Discussion (Page -14) to avoid any overstatement of the prognostic relevance of these individual genes. Specifically, we now clearly distinguish the comparatively stronger association observed for RBSN from the more limited effects seen with the other candidates. Importantly, we have removed language suggesting that these genes could serve as standalone prognostic biomarkers. We further clarify that the survival analyses

are intended as supportive and exploratory, providing clinical context consistent with the functional role of metastasis suppressors identified through our in vivo CRISPR screening approach. We emphasize that the primary contribution of this study lies in the functional identification and in vivo validation of metastasis suppressors, rather than in establishing prognostic biomarkers.

Abstract:”*Integration with patient datasets revealed reduced expression of these genes in tumors and associations with advanced disease, with higher expression trending toward improved outcomes.....*”

Results, page 9, first paragraph: “*Furthermore, we evaluated the association between candidate MSG expression and patient outcomes using Kaplan–Meier survival analyses. Higher expression of VPS45, RBSN, NF2, and CMTR2 was generally associated with trends toward improved relapse-free survival (RFS) and overall survival (OS) across breast cancer cohorts (Fig. 4d, Supplementary Fig. 4c). Among these, RBSN displayed the most consistent and pronounced association with both RFS and OS, whereas VPS45, NF2, and CMTR2 showed more modest and variable trends across datasets. These analyses are exploratory and intended to provide clinical context consistent with the metastasis suppressor functions identified through our in vivo functional screening approach*”.

Fourth paragraph:” *Collectively, these data demonstrate that the identified MSG candidates are consistently downregulated in breast cancer, particularly in TNBC, with reduced expression associated with advanced disease features. Survival analyses provide supportive evidence, with RBSN showing the strongest association with patient outcomes, while the remaining genes exhibit more modest trends. These findings are consistent with a tumor-suppressive role in metastatic progression*”.

Discussion, page14:” *Our findings identify VPS45, CMTR2, RBSN, and NF2 as key regulators of tumor progression and metastasis in TNBC. Analysis of clinical datasets revealed that reduced expression of these four genes is associated with disease progression and poor prognosis in breast cancer patients, particularly those with TNBC. Survival analyses provide limited and exploratory support for clinical association, with RBSN showing the most consistent relationship with patient outcomes, whereas VPS45, NF2, and CMTR2 display more modest and variable trends. These observations are consistent with the metastasis suppressor functions identified here but do not establish these genes as independent prognostic biomarkers*”.

Minor:

1. Supplemental Table 1 reports the genes whose guides were overrepresented in the screen (likely suppressors). It would also be good to report the genes whose guides were underrepresented in the screen (likely enhancers) in a similar Table (or same table) to enable readers to build on this study.

We thank the reviewer for this important suggestion. In response, we have expanded Supplementary Table 1 to include genes whose sgRNAs were underrepresented in the screen (putative metastasis enhancers), alongside the previously reported overrepresented genes (putative suppressors). These are now clearly annotated as the negative selection profile. Genes in the negative selection set are ranked according to the lowest false discovery rate (FDR) derived from sgRNA read counts. To ensure robustness, we report only sgRNAs with a $\log_2$ fold change (LFC) < -2, with top candidates defined by FDR < 0.05. Consistent with the

enriched gene set, the depleted genes are presented with the same metrics (rank, log₂ fold change, *p*-value, and FDR), enabling direct comparison across selection profiles. Supplementary Table 1 therefore now provides a unified resource comprising both positively selected (enriched) and negatively selected (depleted) gene sets. We note that several of the top depleted genes (e.g., *SUMO2*, *DCAF13*, *PKM*, and *TLN1*) were already referenced in the Results section (“Unbiased in vivo genome-wide metastasis suppressor screen”), where prior studies supporting their roles in breast cancer progression and metastasis were cited.

2. Why was the normalization strategy changed for 6D relative to that used in 2C,I?

We thank the reviewer for raising this point and apologize for the lack of clarity in the original submission. The normalization strategy differs between Figures 2C/2I (Figs. 2f/2m in the revised manuscript) and Fig. 6D (Fig. 7d in the revised manuscript), due to distinct metastatic phenotypes observed in the CRISPR knockout versus CRISPR activation settings. In the CRISPR knockout experiments (Figs. 2f, 2m), the metastatic burden was primarily driven by differences in nodule number, while nodule sizes were largely comparable across groups (Figs. 2g, 2n, revised manuscript). Under these conditions, quantification based on nodule counts alone provides an accurate and unbiased measure of metastatic burden. In contrast, in the CRISPR activation experiments (Fig. 7d), activation of the target genes resulted in a marked reduction in metastatic lesion sizes, with control (NTC) animals consistently exhibiting larger nodules (Fig. 7e). As a result, nodule counts alone would underestimate the total metastatic burden and fail to capture biologically meaningful differences. To account for this, we applied the Wexler semi-quantitative scoring method (Wexler, 1996; <https://pubmed.ncbi.nlm.nih.gov/4160686/>), in which each lesion is weighted according to its diameter. This approach integrates both lesion number and size into a single metric, providing a more accurate assessment of metastatic burden under conditions where lesion size varies substantially. This rationale has now been clarified in the Methods (page 22-23) and figure legend (Fig. 7), and the appropriate reference (Wexler paper, 1996) has been added as well. See copies of Figs. 2 and 7 below.

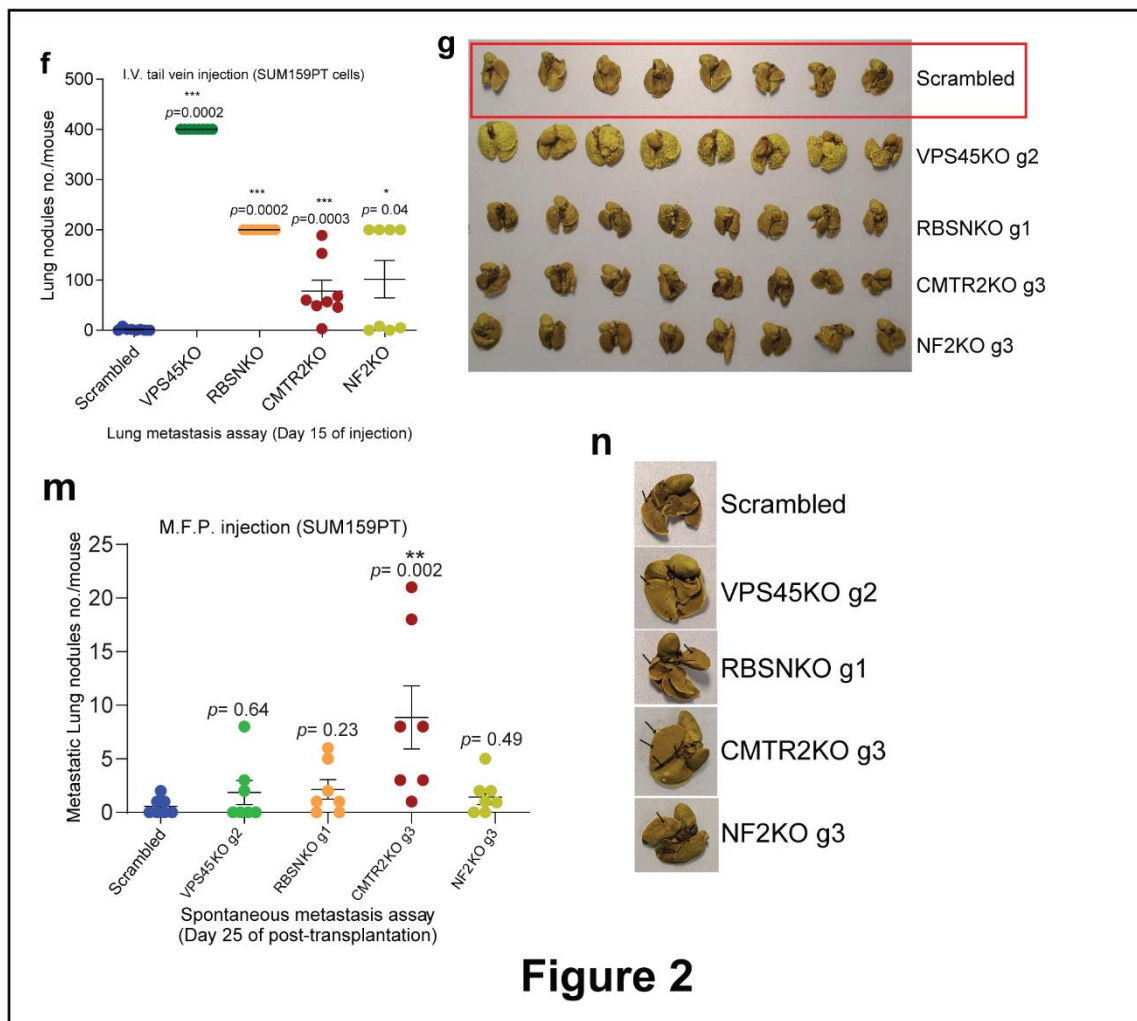


Figure 2

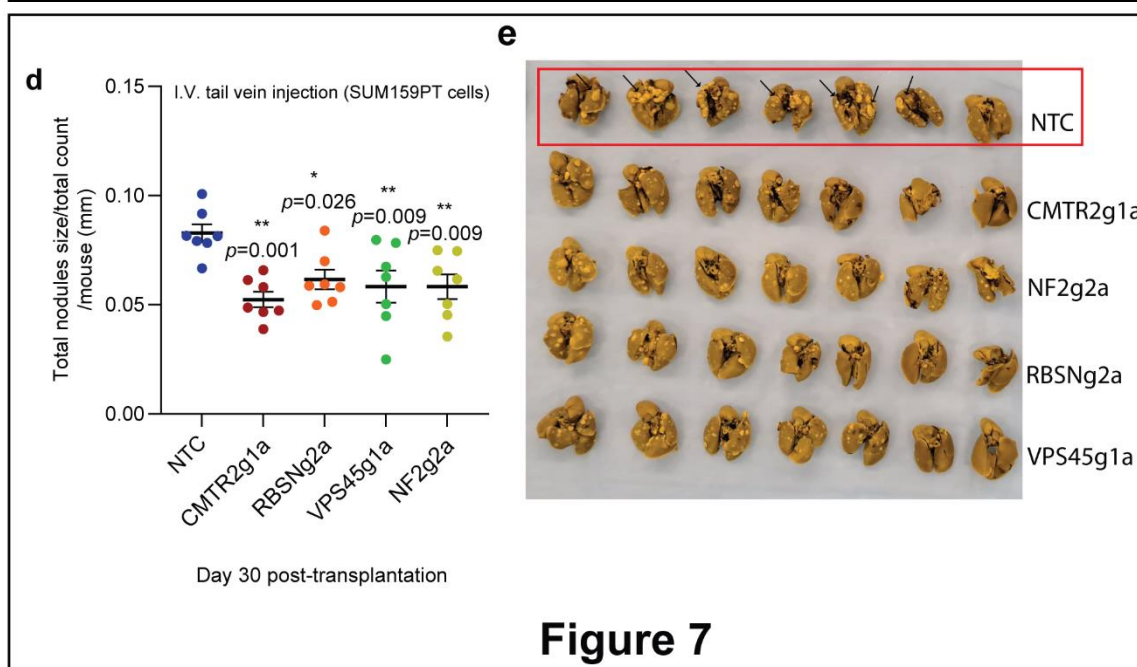
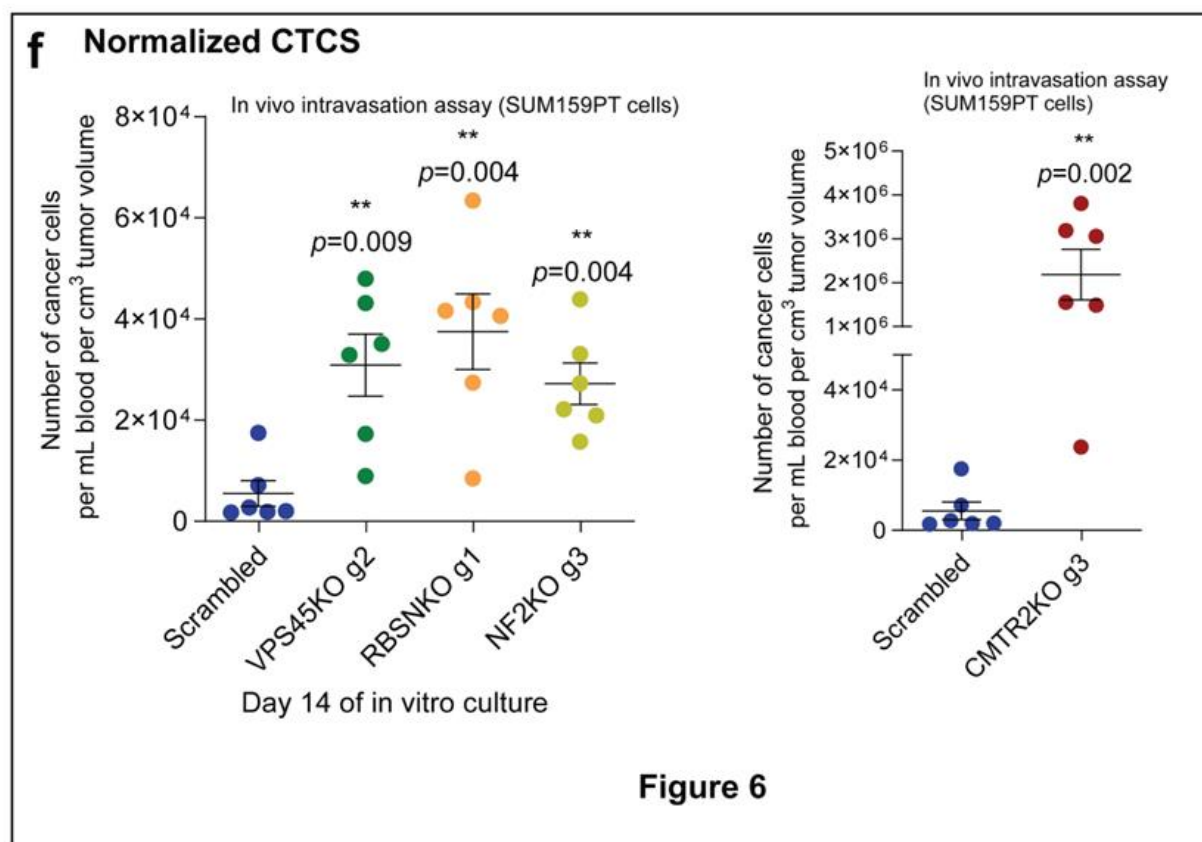


Figure 7

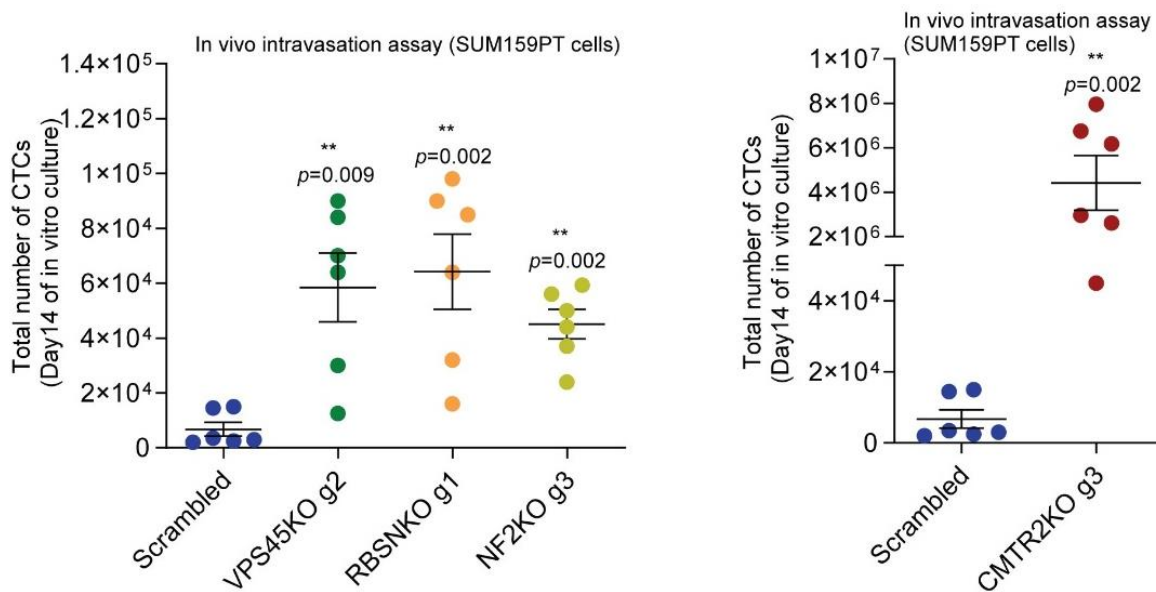
3. What does 5F look like without normalization for tumor size? I understand why they might normalize in that way but some small primary tumors are highly metastatic and some large tumors minimally metastatic, in patients and in experimental models.

We thank the reviewer for raising this important point. While normalization to tumor volume was initially included to account for differences in primary tumor burden (original Fig. 5F, now new Fig. 6f in the revised manuscript, we agree that metastatic propensity does not necessarily scale with tumor size, as small tumors can exhibit high metastatic potential, whereas larger tumors may show limited dissemination.



To address this concern, we reanalyzed the data using raw CTC counts without normalization to tumor volume (shown below, for the reviewer's evaluation). Importantly, the difference between the KO groups and the scrambled control remained statistically significant, indicating that the observed phenotype is not driven by tumor size and is robust across both normalized and non-normalized analyses. As expected, absolute CTC counts were higher in the absence of normalization, consistent with the influence of tumor burden on total CTC output. This supports the rationale for volume normalization when the goal is to assess intrinsic CTC shedding capacity independent of tumor size. Based on these considerations, we have retained the normalized data in Fig. 6f, as it more accurately reflects tumor cell-intrinsic metastatic behavior. However, we appreciate the reviewer's point and hope this clarifies our rationale for the data analyses.

CTCs counts without normalization to the tumor volume



4. BRCA is not a good abbreviation for breast cancer as it is also the abbreviation for one of the main tumor suppressors in breast cancer (e.g. title of Figure 3).

Noted. We agree that the term BRCA is potentially ambiguous, as it is widely used to refer to BRCA1/2 tumor suppressor genes rather than breast cancer more broadly. In response, we have corrected the terminology throughout the UALCAN analysis and all associated figures, replacing “BRCA” with “Breast Cancer” to ensure clarity and avoid potential misinterpretation.

Reviewer #2 (Remarks to the Author):

Galal et al. utilize an unbiased genome-wide in vivo CRISPR/Cas9 screen in a triple-negative breast cancer (TNBC) xenograft model to identify novel metastasis suppressor genes (MSGs). The study identifies and validates VPS45, CMTR2, RBSN, and NF2 as potent suppressors, demonstrating that their ablation promotes epithelial-to-mesenchymal transition (EMT), invasion, and lung colonization, while their endogenous activation significantly abrogates metastatic potential. Notably, the authors highlight a specific role for CMTR2 in maintaining vascular integrity, showing that its loss drives pathological angiogenesis and intra-tumoral heterogeneity that facilitate circulating tumor cell intravasation. These functional findings are corroborated by clinical analyses associating reduced expression of these targets with poor patient prognosis, suggesting they represent a network of clinically relevant barriers to TNBC progression. Here are some concerns to be addressed before publication:

We thank the reviewer for the assessment of our work. We also appreciate the constructive feedback regarding the current limitations and have addressed these points in detail below to strengthen the manuscript.

Major concerns:

1. A key limitation of the immunocompromised NSG mouse model is the absence of immune cell populations. This differs significantly from human cancers, where immunosuppressive cells such as MDSCs can promote metastasis. To address this, the authors should validate the top hits in an immunocompetent mouse model, such as C57BL/6 with the E0771 cancer cell line.

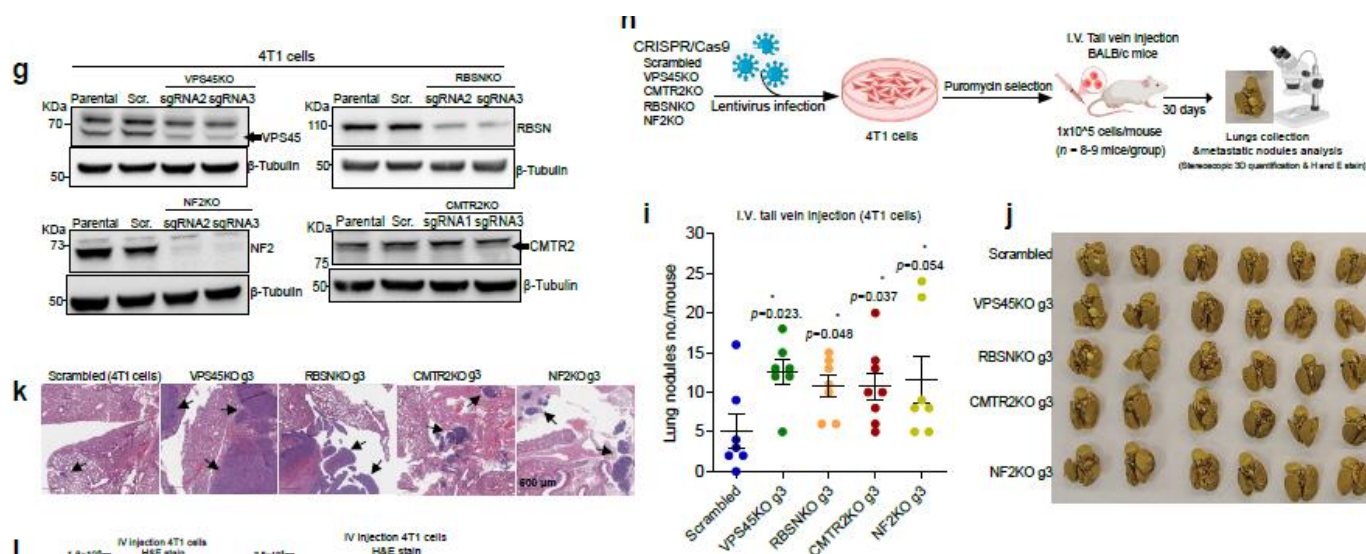
We thank the reviewer for this important comment regarding the limitation of immunocompromised NSG mouse model systems and the contribution of immune populations to metastatic progression. We fully agree that immune components play a critical role in shaping metastatic dissemination in human cancers.

The use of NSG mice in our study was deliberate and aligned with the primary objective of the screen, namely the identification of tumor cell–intrinsic metastasis suppressor genes (MSGs). Immunodeficient models are widely established in this context, as they enable interrogation of cancer cell–autonomous mechanisms while minimizing confounding effects from adaptive immune responses. This approach maximizes sensitivity for detecting intrinsic regulators of metastatic fitness. To ensure robustness and mitigate model-specific bias, our validation strategy incorporated multiple orthogonal systems, including three TNBC cell lines with distinct molecular backgrounds (SUM159PT, MDA-MB-231, and, in this revision, SUM149PT), as well as complementary in vivo approaches (orthotopic implantation and tail vein metastasis assays). The strong concordance of metastatic phenotypes across these models supports a conserved tumor-intrinsic function for the identified genes.

We agree, however, that evaluating these findings in an immunocompetent context is important to understand how tumor-intrinsic programs interact with the immune microenvironment. While the reviewer suggested the E0771 model, it is important to note that while some studies describe the cell line as having basal-like or triple-negative features, E0771 is most commonly classified as a Luminal B–like breast cancer model with variable receptor status reported across studies, and therefore does not fully recapitulate the biology of triple-negative breast cancer. To more appropriately address this point, we selected the 4T1 syngeneic model, which is a well-established and highly aggressive TNBC system in immunocompetent BALB/c mice. The 4T1 model closely mimics key features of human TNBC, including spontaneous metastasis and the presence of a complex, immunosuppressive microenvironment enriched in MDSCs and other myeloid populations.

To directly address the reviewer's concern, we extended our analysis to an immunocompetent setting using the 4T1 syngeneic model. We performed a comprehensive series of metastasis assays, including experimental lung colonization (intravenous injection) and spontaneous metastasis following orthotopic mammary fat pad implantation. Multiple independent CRISPR knockout constructs were generated for each target gene to ensure efficient and robust gene silencing in the mouse context (see new Fig. 3) and screen shot below.

In the experimental metastasis assay (tail vein injection; 1×10^5 cells; $n = 8-9$ mice per group), loss of VPS45, RBSN, CMTR2, or NF2 resulted in a consistent and statistically significant increase in lung metastatic burden compared to control cells (all $p < 0.05$). Quantification of metastatic nodules revealed an increase from 5.14 ± 2.10 in controls to 12.57 ± 1.49 (VPS45-KO), 10.71 ± 1.38 (RBSN-KO), 10.75 ± 1.72 (CMTR2-KO), and 11.57 ± 3.02 (NF2-KO) (mean $\pm$ SEM), corresponding to an approximately two-fold enhancement in metastatic colonization efficiency. Among these, VPS45 loss elicited the most pronounced phenotype (see new Fig.3) and screen shot below.



We next evaluated metastatic progression in a spontaneous metastasis model using orthotopic mammary fat pad transplantation (2×10^5 cells; $n = 8$ mice per group). Under these more physiologically stringent and immune-competent conditions, loss of all four genes significantly enhanced lung metastasis (all $p < 0.01$). Control tumors exhibited minimal metastatic burden (3.14 ± 1.28 nodules), whereas NF2-KO (12.00 ± 1.79), RBSN-KO (13.57 ± 2.78), and CMTR2-KO (15.29 ± 2.97) showed marked increases. Notably, VPS45-KO again displayed a striking phenotype (41.14 ± 18.70 nodules), suggesting a dominant role in constraining metastatic dissemination (see new Fig. 3). Importantly, primary tumor growth was not consistently altered across groups, as assessed by longitudinal measurements and two-way repeated-measures ANOVA. Although transient differences were observed at isolated time points, no sustained effect on tumor growth was detected. In contrast, metastatic burden was robustly and reproducibly increased across all knockout conditions. These findings indicate that these genes

preferentially regulate metastatic competence rather than primary tumor proliferation, a defining characteristic of metastasis suppressor genes (see new Fig. 3) and screen shot below.

Collectively, these results demonstrate that the pro-metastatic effects associated with VPS45, RBSN, CMTR2, and NF2 loss are preserved in an immunocompetent context, despite the presence of an intact immune system and tumor-immune interactions. This strongly supports

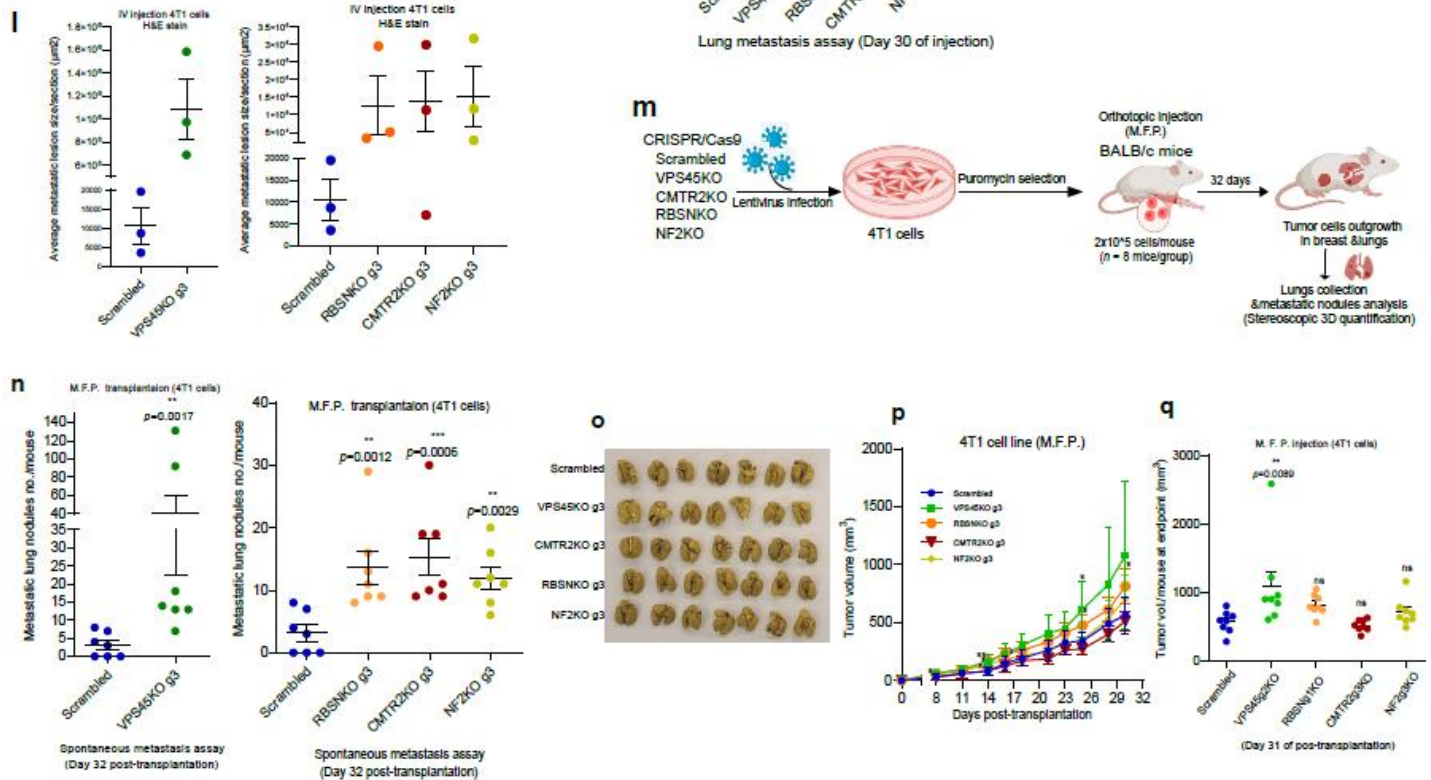


Figure 3

the conclusion that these genes function predominantly through tumor-intrinsic mechanisms while remaining fully operative in physiologically relevant settings. The inclusion of these syngeneic data substantially strengthens the translational relevance of our study and directly addresses the reviewer's concern. These results have been incorporated into the revised manuscript (new full Fig. 3), and the Results and Discussion sections have been updated accordingly.

Results, pages 6-7: See full new section and Fig.3: “Loss of VPS45, RBSN, CMTR2, NF2 promotes metastasis across immunodeficient and immunocompetent TNBC models. To determine whether these metastasis suppressor genes (MSGs) retain their function in the presence of an intact immune system, we next employed the 4T1 syngeneic model in immunocompetent BALB/c mice. Efficient gene disruption in 4T1 cells was confirmed by immunoblotting (Fig. 3g). In experimental metastasis assays (tail vein injection; Fig. 3h), loss of VPS45, RBSN, CMTR2, or NF2 resulted in a consistent and statistically significant increase in lung metastatic burden compared to control cells (all $p \leq 0.05$) (Fig. 3i, j). Specifically, metastatic nodules increased from 5.14 ± 2.10 in controls to 12.57 ± 1.49 (VPS45-KO), 10.71 ± 1.38 (RBSN-KO), 10.75 ± 1.72 (CMTR2-KO), and 11.57 ± 3.02 (NF2-KO) (mean $\pm$ SEM),

corresponding to approximately two-fold increases in metastatic colonization efficiency, with VPS45 loss producing the strongest effect. Histopathological analysis further confirmed a significant increase in both the number and area of metastatic lung lesions across all knockout groups (**Fig. 3k, l**).

Consistent results were obtained in a spontaneous metastasis setting using orthotopic mammary fat pad transplantation (**Fig. 3m-o**), where loss of all four genes significantly enhanced lung metastasis (all $p < 0.01$). Control tumors exhibited minimal metastatic burden (3.14 ± 1.28 nodules), whereas NF2-KO (12.00 ± 1.79), RBSN-KO (13.57 ± 2.78), and CMTR2-KO (15.29 ± 2.97) showed marked increases. VPS45-KO again displayed a striking phenotype (41.14 ± 18.70 nodules), suggesting a dominant role in constraining metastatic dissemination. Notably, primary tumor growth was not consistently affected across groups, as assessed by longitudinal measurements and two-way repeated-measures ANOVA (**Fig. 3p**), indicating that these genes preferentially regulate metastatic competence rather than primary tumor proliferation. A modest but statistically significant increase in tumor growth was observed at endpoint in the VPS45-KO group (one-way ANOVA; **Fig. 3 q**), although this effect was not sustained over time.

Collectively, these findings demonstrate that the metastasis-suppressive functions of VPS45, RBSN, CMTR2, and NF2 are preserved across both immunodeficient and immunocompetent settings. This cross-model consistency strongly supports a tumor-intrinsic role for these genes in restraining metastatic progression while confirming their relevance within a physiologically intact tumor-immune microenvironment”.

Discussion section, page 14: “While our primary screening strategy was designed to identify tumor cell-intrinsic regulators of metastasis using immunodeficient models, it is well established that the tumor microenvironment, particularly immune components, plays a critical role in metastatic progression. To address this, we extended our validation to an immunocompetent setting using the 4T1 syngeneic model. Notably, loss of VPS45, RBSN, CMTR2, and NF2 consistently enhanced metastatic dissemination even in the presence of an intact immune system. These findings indicate that the metastasis-suppressive functions of these genes are predominantly tumor-intrinsic and remain operative despite complex tumor-immune interactions. Importantly, the preservation of these phenotypes across both experimental (tail vein) and spontaneous (orthotopic) metastasis models further underscores their role across multiple steps of the metastatic cascade, including colonization and dissemination. Together with the concordant results observed across independent human TNBC cell lines, these data support a model in which VPS45, RBSN, CMTR2, and NF2 function as core barriers to metastatic competence, with effects that are robust to both genetic background and immune context. This cross-model consistency substantially strengthens the physiological and translational relevance of our findings.

Interestingly, an ongoing investigation using the liposomal CMTR2 inhibitor AT-1965 reported reduced primary tumor growth in orthotopic 4T1 models, an effect attributed to enhanced innate immune sensing of hypomethylated mRNA caps. In contrast, our genetic ablation of CMTR2 did not suppress primary tumor growth but instead promoted metastatic dissemination in both experimental and spontaneous metastasis settings. While these differences may reflect distinct biological consequences of acute pharmacological inhibition versus stable genetic loss, as well as potential immune-mediated effects, they highlight the context-dependent roles of CMTR2.

These observations suggest that therapeutic strategies targeting CMTR2 will require careful evaluation, particularly with respect to their potential impact on metastatic progression”.

2. Based on the screening design, the observed increase in lung tumor nodules could result from a combination of cancer cell seeding, proliferation, and migration. To clarify the underlying mechanism, the authors should quantify the proliferation rate for each CRISPR knockout line. We thank the reviewer for this important point. We agree that increased lung tumor burden could reflect a combination of altered proliferation, survival, and metastatic efficiency, and we therefore systematically assessed whether intrinsic proliferative capacity is altered in all our engineered CRISPR knockout (KO) lines.

To address this, we performed multiple in vitro assays to evaluate proliferation and growth kinetics in SUM159 TNBC cells. MTT and SRB assays revealed that VPS45KO, RBSNKO, and NF2KO cells display no increase in cell growth activity or net cell viability compared to scrambled controls, while CMTR2KO exhibited a modest reduction (~17%) in viability. Consistently, SRB-based total biomass measurements showed a small but statistically significant decrease across all four KOs, indicating no intrinsic growth advantage. At the clonal level, colony formation assays demonstrated comparable colony numbers between all 4 KOs and control cells, indicating that the ability of single cells to undergo sustained proliferative expansion is not enhanced. In line with these findings, Western blot analysis of the proliferation marker PCNA showed no increase in expression in any of the KO lines, further supporting the absence of increased cell cycle progression (see new results below).

Together, these orthogonal assays consistently demonstrate that loss of these genes does not enhance intrinsic proliferative capacity and may, in some cases, slightly impair growth under standard culture conditions.

Importantly, the metastatic phenotype is preserved in experimental metastasis (tail-vein) models, where primary tumor growth is bypassed. This provides strong functional evidence that the increased lung colonization is not driven by differences in primary tumor proliferation but rather reflects enhanced metastatic efficiency. Furthermore, the magnitude of the increase in lung nodules substantially exceeds any minor differences observed in primary tumor growth, arguing against proliferation as the dominant driver.

While we cannot fully exclude context-dependent effects of the in vivo microenvironment on tumor expansion, the collective data strongly support a model in which loss of these genes enhances key steps of the metastatic cascade, including dissemination, survival in circulation, and colonization of the lung microenvironment, rather than increasing intrinsic proliferation. To further ensure transparency, all proliferation-related data have been included in the revised Supplementary Information (**new Supplementary Fig. 3**). In addition, we have clarified this point in the manuscript to emphasize that the dominant phenotype associated with these perturbations is increased metastatic fitness rather than enhanced proliferation.

Result section, page 6: “To directly address whether the increased lung tumor burden observed upon gene loss is driven by altered proliferation, we systematically evaluated the intrinsic growth capacity of the KO lines. Across multiple orthogonal assays, including MTT and SRB measurements of cell growth and viability, as well as colony formation assays assessing clonal expansion, we observed no increase in proliferative capacity in any of the KO cells relative to

controls. Consistently, expression of the proliferation marker PCNA remained unchanged. In contrast, some KOs exhibited modest reductions in viability and biomass, indicating that loss of these genes does not confer a growth advantage under standard conditions (Supplementary Fig. 3a-d). Importantly, the enhanced metastatic phenotype was maintained in experimental metastasis (tail-vein) models, where primary tumor growth is bypassed, further supporting a proliferation-independent mechanism. Collectively, these data indicate that the dominant effect of these perturbations is an increase in metastatic fitness rather than enhanced intrinsic proliferation”.

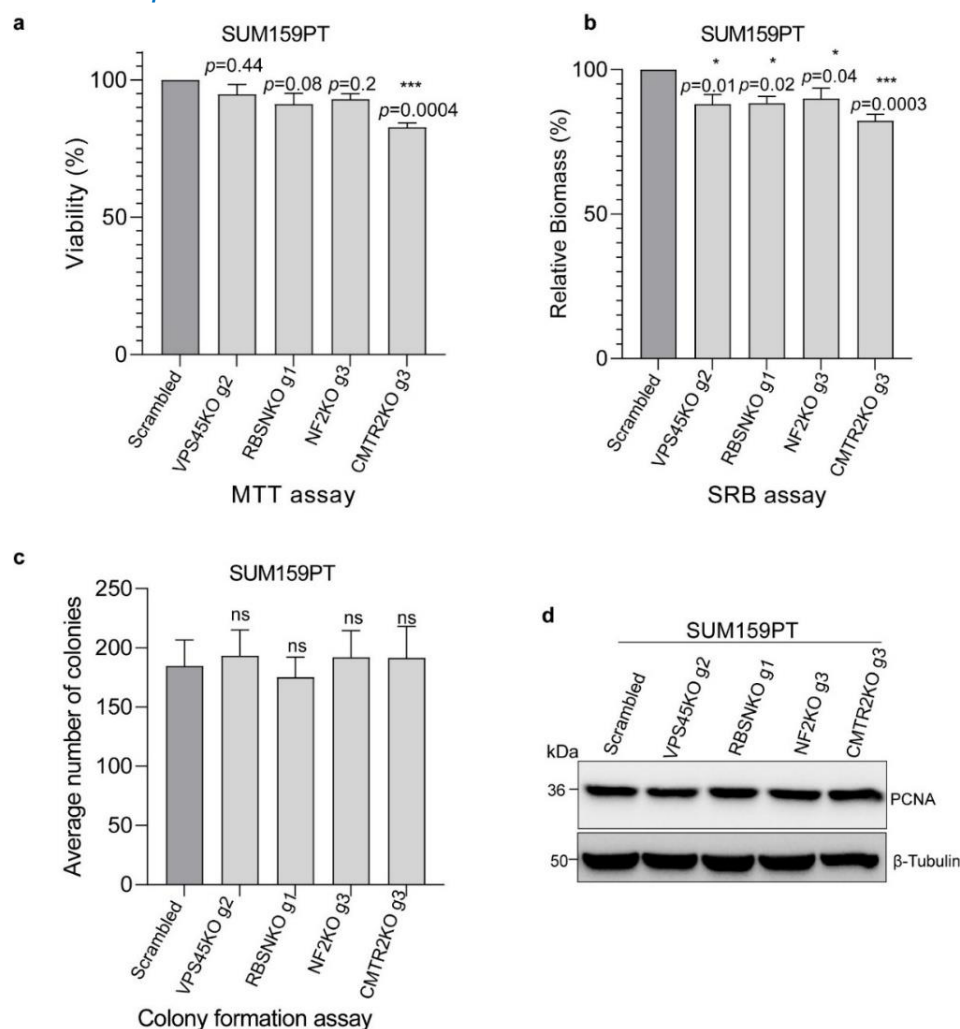


Figure legend for Supplementary Fig. 3. **Loss of MSGs does not enhance intrinsic proliferative capacity in SUM159PT cells.** a, b MTT and SRB assays show no increase in cell growth and viability in VPS45KO, RBSNKO, NF2KO, and CMTR2KO cells relative to scrambled controls, with modest reductions observed in some KOs. c Colony formation assays reveal no significant differences in clonogenic potential across all groups. d Immunoblot analysis of proliferating cell nuclear antigen (PCNA) expression in knockout and control cells confirms unchanged proliferation marker expression. (n = 3 replicates), data are presented as mean ± SEM; statistical significance was

determined by one-way ANOVA, as indicated (ns, not significant).

3. It would be interesting to explore potential interactions among these four genes. For example, does simultaneous knockout of two of the four genes produce a synergistic effect on tumor metastasis? Such an analysis could provide valuable insight into critical gene–gene interactions and whether they are on the same pathway or not.

We thank the reviewer for this insightful suggestion. We agree that defining gene–gene interactions is critical to understanding whether the identified metastasis suppressors function within shared or independent pathways.

To address this, we performed systematic pairwise double knockout experiments among the four target genes (*VPS45*, *RBSN*, *NF2* and *CMTR2*) using CRISPR/Cas9 in SUM159 cells. Efficiency of single and double KO were validated at the protein expression level by immunoblotting (see **new Fig. 6h**, below). Functional assessment using serum-free scratch assays revealed that, among all combinations tested, only the simultaneous loss of *VPS45* and *RBSN* resulted in an enhancement of cell migration, significantly exceeding both single knockouts and controls (ANOVA, $p < 0.0001$). Importantly, the effect followed a stepwise pattern (control < single KO < double KO), consistent with a cooperative (greater-than-additive) interaction between *VPS45* and *RBSN* (see **new Fig. 6i**, below). Consistent with this phenotype, at the molecular level, the *VPS45*/*RBSN* double knockout also induced a marked upregulation of *MMP1* mRNA expression, supporting a mechanistic link between this gene–gene interaction and enhanced invasive behavior (see **new Fig. 6j**, below).

To then determine whether this functional cooperation reflects a shared molecular pathway, we performed co-immunoprecipitation analyses. These experiments confirmed a robust endogenous interaction between *VPS45* and *RBSN*, whereas no interaction was detected among other pairwise combinations. Moreover, *VPS45* loss reduced *RBSN* protein levels, consistent with a role for *VPS45* in maintaining *RBSN* stability, and in agreement with previous reports demonstrating *VPS45*–*RBSN* interaction and regulation of *RBSN* abundance in HeLa cells (PMID: 19931244). Together with prior evidence implicating both proteins in endosomal trafficking, these findings support a model in which *VPS45* and *RBSN* function as a coordinated complex regulating vesicular trafficking and metastasis suppression (see **new Fig. 6k**, below). In contrast, no synergistic effects or physical interactions were observed for combinations involving *NF2* or *CMTR2*. This suggests that these genes likely act through independent pathways, consistent with their known roles in Hippo signaling (*NF2*) and RNA processing (*CMTR2*).

Collectively, these results indicate that the four metastasis suppressors identified in our screen operate through both shared (*VPS45*/*RBSN*) and distinct (*NF2*, *CMTR2*) mechanisms, highlighting the convergence of multiple biological processes in the regulation of tumor metastasis.

These new data have been incorporated into the revised manuscript (**New Figs. 6h–k**) and relevant descriptions have been added to the Results section, page 11: “*To investigate potential functional interactions among the identified metastasis suppressors, we performed systematic pairwise double knockout experiments in SUM159 cells. Immunoblot validation confirmed efficient single and double gene disruption (Fig. 6h). Among all combinations tested, only the simultaneous loss of VPS45 and RBSN resulted in a marked increase in cell migration, as assessed by wound-healing assays under serum-free conditions (Fig. 6i). Notably, the effect followed a stepwise increase from control to single knockouts to double knockout, consistent with a cooperative interaction between VPS45 and RBSN. At the molecular level, VPS45/RBSN double knockout induced a significant upregulation of MMP1 expression compared to single knockouts (Fig. 6j), supporting a mechanistic link to enhanced invasive potential. Co-immunoprecipitation assays revealed a robust endogenous interaction between VPS45 and RBSN in SUM159 cells (Fig. 6k). Furthermore, VPS45 depletion reduced RBSN protein levels, suggesting that VPS45 contributes to RBSN stability. No interactions were detected among other gene pairs.*”). Together, these results identify a specific *VPS45*–*RBSN* functional axis that

cooperatively regulates cell motility and invasive gene expression, whereas NF2 and CMTR2 appear to act through independent pathways”.

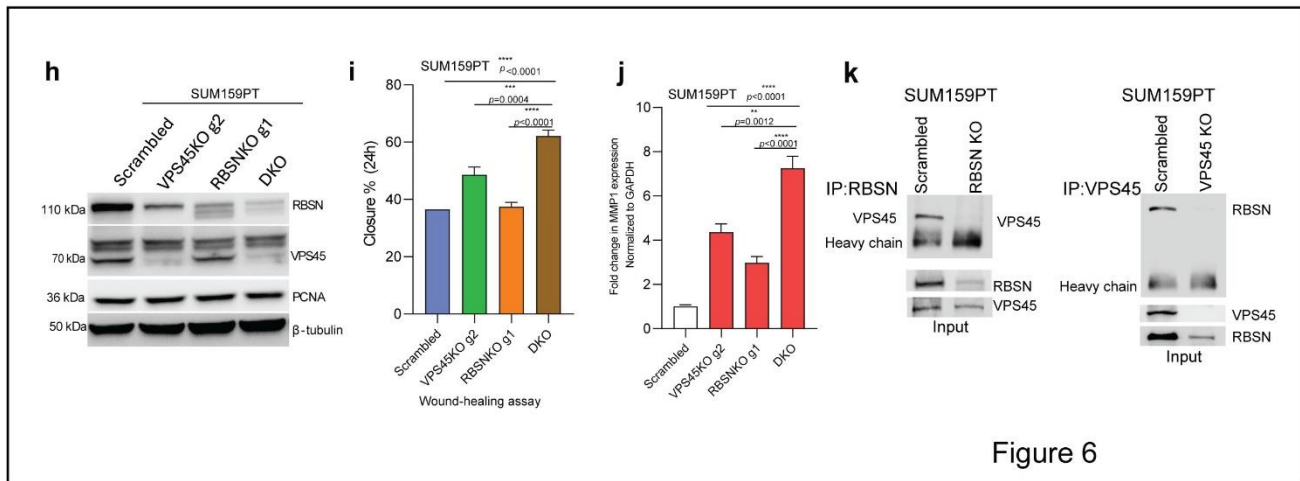


Fig. 6. h-k VPS45 interacts with RBSN in TNBC cells. **h** Validation of VPS45 and RBSN single and double knockout. Immunoblot analysis of VPS45 and RBSN expressions in SUM159 cells following CRISPR/Cas9-mediated single knockout (VPS45KO, RBSNKO) or double knockout (DKO). PCNA and β -tubulin serve as loading controls. **i** VPS45 and RBSN cooperatively suppress cell migration. Wound-healing assay performed in SUM159 cells under serum-free conditions to assess cell migration. Quantification of wound closure at 24 h is shown for control (scrambled), VPS45KO, RBSNKO, and VPS45/RBSN double knockout (DKO). Data are presented as mean $\pm$ SEM ($n = 3$ replicates). Statistical significance was determined by one-way ANOVA with multiple comparisons; exact p -values are indicated. **j** VPS45/RBSN double knockout induces MMP1 expression. qRT-PCR analysis of MMP1 mRNA expression in SUM159 cells following single or double knockout as indicated. Expression levels were normalized to GAPDH and are presented as fold change relative to control. Data are shown as mean $\pm$ SEM ($n = 3$ replicates). Statistical significance was determined by one-way ANOVA with multiple comparisons; exact p values are indicated. **k** Co-immunoprecipitation of endogenous VPS45 and RBSN in SUM159 cells. Immunoprecipitation (IP) was performed using anti-RBSN or anti-VPS45 antibodies, followed by immunoblotting as indicated. Reciprocal pulldown confirms VPS45–RBSN interaction. Input controls are shown.

Minor concerns:

1. The Surveyor Nuclease Assay shown in Figure 2A lacks professionalism. The authors should invert the image colors, as the current black background with white bands makes interpretation difficult. In addition, the authors should quantify the editing efficiency. Next-generation sequencing (NGS) is considered the gold standard for assessing gene-editing outcomes; therefore, targeted sequencing should be performed to accurately determine the INDEL frequency.

We thank the reviewer for this important comment regarding the presentation and quantification of genome editing efficiency. The Surveyor nuclease assay is a widely used and reliable method for initial assessment of CRISPR-mediated genome editing, as it enables rapid detection of heteroduplex formation and provides a semi-quantitative estimate of INDEL formation. In the

revised manuscript, as suggested by the reviewer, we have improved the presentation of this assay by updating the gel image for enhanced clarity besides the densitometric quantification of editing efficiency (**new Fig. 2b**).

To address the reviewer's point regarding precise quantification, we have further strengthened our analysis by performing targeted amplicon sequencing of edited loci. Genomic DNA from VPS45, RBSN, NF2, and CMTR2 knockout SUM159 cells, along with non-targeting controls, was subjected to Oxford Nanopore Technologies (ONT) sequencing. Full-length reads were analyzed using CRISPResso2, enabling single-base resolution characterization of CRISPR-induced INDELs. Editing efficiencies, calculated relative to scrambled controls, reached 93.17% (VPS45), 97.44% (RBSN), 98.83% (NF2), and 66.30% (CMTR2), demonstrating robust genome editing across all targets (see **new Figs. 2c, d**). Importantly, these modified reads are enriched precisely at the predicted Cas9 cleavage site, as shown by nucleotide distribution patterns surrounding the sgRNA target regions, confirming that the detected sequence alterations are on-target CRISPR-induced INDELs rather than random sequencing noise. To provide a comprehensive view of editing outcomes, we have included detailed CRISPResso2 analyses in **new Supplementary Fig. 2**, including indel size distributions, positional mutation maps, and allele frequency profiles. These data collectively offer high-resolution insight into editing patterns and confirm consistent, NHEJ-driven mutational signatures across all targeted genes. Accordingly, the Results (**Fig. 2 and Supplementary Fig. 2**), Methods (Targeted amplicon sequencing and CRISPR editing analysis), and Discussion sections have been revised to reflect these additions. Together, these improvements replace reliance on qualitative assessment with robust, sequencing-based quantification of genome editing efficiency.

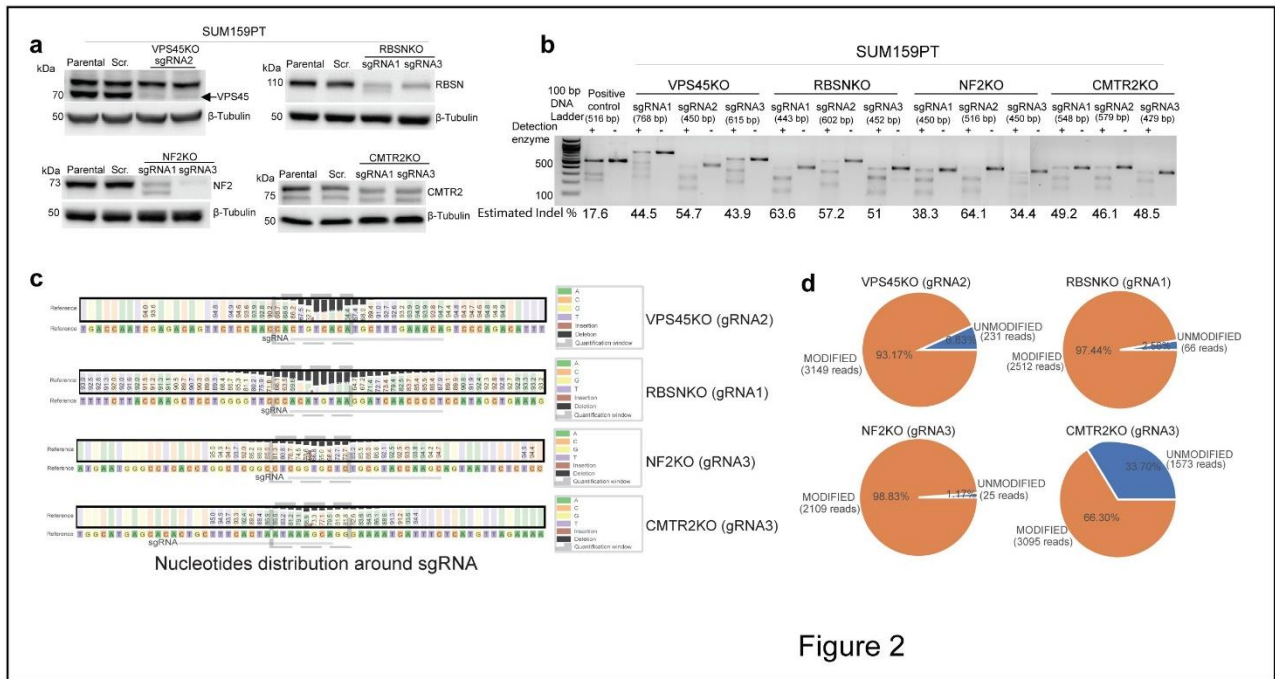
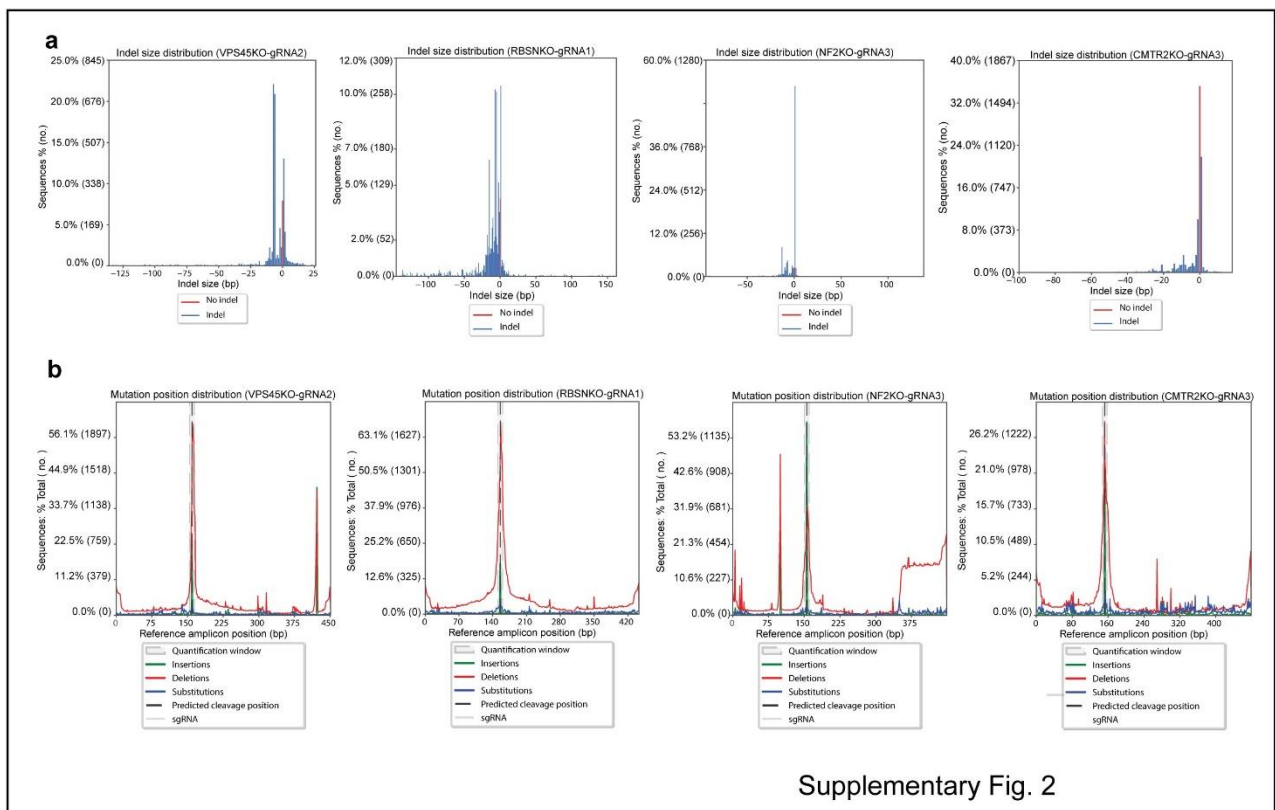


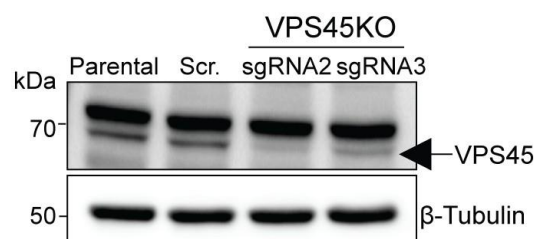
Figure 2



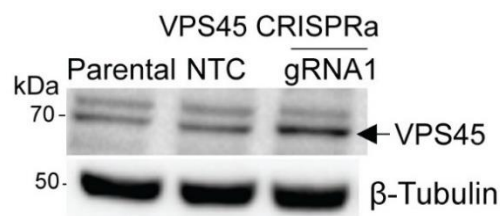
Supplementary Fig. 2

2. In the western blot image presented in Figure 2A, the knockout of VPS45 is not clearly demonstrated. Additionally, the identity of the more prominent band at the top is unclear and should be explained.

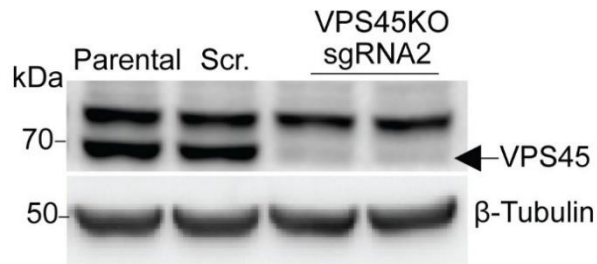
We thank the reviewer for this comment regarding the interpretation of the VPS45 western blot in Fig.2A. The VPS45 antibody used (Proteintech, 12006-1-AP) is a polyclonal antibody that can generate non-specific cross-reactive signals due to recognition of multiple epitopes. Consistent with this, two bands were detected; however, only the ~70 kDa band was specifically lost upon VPS45 knockout, whereas the higher molecular weight band remained unchanged.



Similarly, in CRISPR activation (CRISPRa) of VPS45 in the SUM159 cell line, CRISPRa specifically increased the intensity of the ~70 kDa band, while the higher molecular weight band remained unaffected. This reciprocal gain-of-function evidence provides genetic confirmation that the ~70 kDa band corresponds to VPS45 protein.



That said, to further validate antibody specificity, we obtained an independent VPS45 antibody (Proteintech, 12616-1-AP) and reanalyzed protein lysates from SUM159 cells. This antibody recapitulated the same pattern, showing selective loss of the ~70 kDa band in VPS45 knockout cells, while the higher molecular weight band remained unchanged. Collectively, the concordant results observed across CRISPRa and CRISPR knockout conditions confirm that the ~70 kDa band corresponds to VPS45, whereas the higher molecular weight band likely reflects non-specific antibody binding. For clarity, we have updated the western blot Fig. 2A with the new figure obtained using the new antibody, VPS45 antibody (Proteintech, 12616-1-AP), as shown below.



3. In the discussion section, the authors should elaborate on the therapeutic implications of identifying these four gene targets. For instance, how might one selectively activate or restore the expression of these genes in TNBC?

We thank the reviewer for this insightful comment regarding the therapeutic implications of these metastasis suppressor genes (MSGs). The identification of VPS45, RBSN, NF2, and CMTR2 as regulators of metastatic progression in TNBC suggests several mechanistically distinct, yet complementary, avenues for therapeutic intervention aimed at restoring their functional activity.

One potential strategy involves transcriptional reactivation of endogenous gene expression. Emerging approaches such as small activating RNAs (saRNAs)^{1,2} have shown the capacity to selectively upregulate target genes without genomic integration and are currently under preclinical and early translational investigation. While not yet clinically established in oncology, such approaches provide a conceptual framework for reinstating MSG expression.

In addition, our analyses suggest that post-transcriptional regulation may contribute to CMTR2 suppression. In silico prediction (TargetScan 8.0) identifies CMTR2 as a high-confidence target of hsa-miR-9-5p, a microRNA previously implicated in promoting breast cancer metastasis³. This raises the possibility that inhibition of miR-9-5p using antagomirs or LNA-modified anti-miRs could relieve repression of CMTR2 and restore its antimetastatic function. Although this interaction remains to be experimentally validated, it highlights a plausible and therapeutically tractable regulatory axis.

Beyond modulation of endogenous regulatory mechanisms, emerging RNA- and gene-based delivery platforms offer additional opportunities for functional restoration. For example, lipid nanoparticle (LNP)-mediated delivery of synthetic mRNA has recently demonstrated clinical feasibility and could, in principle, enable transient re-expression of these metastasis suppressors. Such approaches may be particularly attractive in the context of metastasis, where restoring regulatory network balance rather than inhibiting a single oncogenic pathway may be required.

Importantly, the mechanistic diversity of these genes, spanning vesicular trafficking (VPS45, RBSN), cytoskeletal regulation (NF2), and mRNA cap modification (CMTR2), suggests that their restoration could impact multiple, non-redundant aspects of the metastatic cascade. Collectively, these findings support a model in which therapeutic strategies aimed at reinstating MSG function may provide a complementary approach to conventional pathway inhibition, although further work will be required to translate these concepts into clinically actionable interventions.

New Discussion Paragraph, last section, pages 16-17:

“Building on this framework, the identification of VPS45, RBSN, NF2, and CMTR2 as metastasis suppressor genes (MSGs) in TNBC highlights several potential translational avenues for restoring their functional activity. Approaches aimed at reactivating endogenous gene expression, including small activating RNAs (saRNAs)^{1,2}, are under active preclinical investigation and may provide a strategy to reinstate MSG expression. In parallel, post-transcriptional regulation by oncogenic microRNAs may represent an additional layer of control; for instance, CMTR2 is predicted to be a target of hsa-miR-9-5p, a microRNA implicated in breast cancer metastasis³, suggesting that anti-miR-based strategies could relieve repression and restore its function. Furthermore, RNA-based delivery platforms, including lipid nanoparticle (LNP)-mediated mRNA delivery, have demonstrated clinical feasibility and may offer a framework for transient protein restoration. The functional diversity of these genes,

spanning vesicular trafficking (VPS45, RBSN), cytoskeletal regulation (NF2), and mRNA cap modification (CMTR2), underscores their involvement in distinct regulatory nodes of metastatic progression. This raises the possibility that restoring their activity could exert broader control over metastatic competence than single-pathway targeting approaches, although such strategies remain to be validated in future studies”.

Reviewer #3 (Remarks to the Author):

The manuscript titled Genome-wide in vivo CRISPR screen identifies CMTR2 as a potent suppressor of metastasis in Triple-negative breast cancer presents data and evidence on the role of four tumor suppressor genes (TSGs) (VPS45, RBSN, CMTR2, and NF2) in TNBC tumor growth, lung metastasis, EMT, and angiogenesis. Notably, the study highlights CMTR2 as a key modulator of TNBC metastasis among these TSGs. The work is well-designed and offers novel insights into the functions of these genes. However, there are certain limitations in fully validating the findings based on the data provided in the current version of the manuscript.

We thank the reviewer for the positive and thoughtful assessment of our work. We also value the reviewer’s constructive feedback regarding the current limitations and have addressed these points in detail below to further strengthen the manuscript (see below).

1. The primary limitation of this study is that all cell culture experiments were conducted using a single TNBC cell line, SUM159. Findings based on one cell line are generally insufficient for publication because they lack reproducibility and generalizability. Incorporating multiple cell lines is crucial to confirm that the observed effects are not cell line specific and to strengthen the validity and broader applicability of the results. Therefore, it is essential to perform key experiments using at least one additional TNBC cell line.”

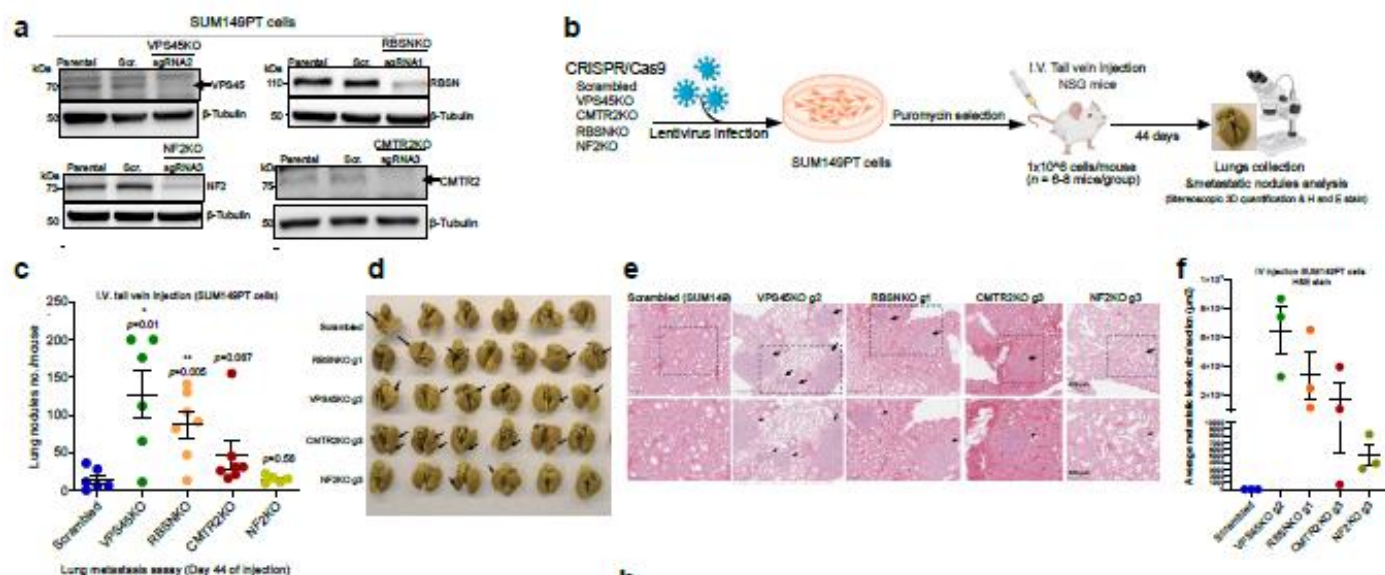
We thank the reviewer for this thoughtful comment regarding the use of multiple TNBC models to ensure robustness and generalizability and avoid the limitation of using a single cell line. We would like to respectfully clarify that our study does not rely on a single cell line. In addition to the SUM159 models (with both tail vein and orthotopic transplantation experiments used for knockout (KO) studies) (Fig. 2), we also conducted gene activation experiments not only using SUM159 cell line (Fig. 7), but also, we performed spontaneous metastasis assay using an additional TNBC cell line, the MDA-MB-231 model (Supplementary Fig. 7), thereby already incorporating multiple experimental systems.

That said, to further strengthen the generalizability of our findings, as suggested by the reviewer, we extended our in vivo analyses to a third independent TNBC model, SUM149PT, derived from inflammatory breast cancer and harboring a BRCA1 mutation. Using a tail vein metastasis assay, we evaluated the effect of KO of VPS45, RBSN, CMTR2, and NF2 compared to a scrambled control.

Consistent with our findings in SUM159, loss of VPS45 and RBSN in SUM149PT cells led to a marked increase in lung metastatic burden, with average lung nodule counts of 127.3 and 86.9, respectively, compared to 14.5 in controls ($p < 0.05$). CMTR2 KO also increased metastatic burden (43.7 vs. 14.5), although this did not reach statistical significance ($p = 0.067$), likely reflecting biological variability and limited sample size. NF2 KO exhibited a more modest effect (14.8 nodules; $p = 0.56$), which may be attributable to model-specific heterogeneity. Importantly, the overall trend across all four genes remained consistent with the metastatic phenotypes observed in SUM159 (new Fig. 2f). Representative images of lung metastases are provided, and metastatic burden was quantified using QuPath software. Total tumor area and average

nodule size were calculated across three serial lung sections (proximal, middle, distal). Collectively, these results demonstrate that our findings are reproducible across multiple independent TNBC models, including SUM159, MDA-MB-231, and SUM149PT. This multi-model validation supports the robustness and broader applicability of our conclusions, rather than a cell line–specific effect. The new SUM149PT data are presented in **new Figs. 3a–f** (see screenshot below).

The results section, pages 6-7 has been modified to include these new findings:” **Loss of VPS45, RBSN, CMTR2, NF2 promotes metastasis across immunodeficient and immunocompetent TNBC models. To mitigate potential cell line–specific effects, we extended**



functional validation to the SUM149PT model, a well-established TNBC system characterized by genomic instability and mutations in key tumor suppressors, including CDKN2A, BRCA1, and TP53 (Cellosaurus accession: RRID: CVCL_3422). Efficient gene knockout was confirmed by immunoblotting (**Fig. 3a**). In tail vein xenograft assays, terminated 44 days post-injection, loss of VPS45, RBSN, CMTR2, and NF2 led to an overall increase in lung metastatic burden (**Fig. 3b–d**). VPS45-KO and RBSN-KO exhibited the most pronounced and statistically significant effects ($p \leq 0.01$), whereas CMTR2-KO showed a consistent but non-significant trend ($p = 0.067$, two-tailed Mann–Whitney U test), and NF2-KO had a modest, non-significant effect ($p = 0.56$). Importantly, these results closely mirror those obtained in SUM159 models, indicating that the observed phenotypes are reproducible across independent TNBC systems with distinct genetic backgrounds.

To provide orthogonal validation of metastatic burden, hematoxylin and eosin (H&E) staining was performed on lung sections ($n = 3$ sections per lung per mouse). Quantification of metastatic nodule area confirmed the increase in metastatic burden across knockout conditions, consistent with nodule count–based analyses (**Figs. 3 e, f**). Together, these data demonstrate that the pro-metastatic effects of VPS45, RBSN, CMTR2, and NF2 loss are conserved across multiple TNBC models, supporting a context-independent role in regulating metastatic progression”.

2. In Figure 2H, the primary tumor size appears larger in the NF2 KO group, whereas lung metastasis is higher in the CMTR2 KO group. This observation aligns with Figure 3B, which

shows a significant decrease in NF2 expression in TNBC compared to the normal group. Please discuss this point and provide a possible explanation for this finding

We thank the reviewer for this insightful observation. While NF2 knockout (KO) in the SUM159 orthotopic model (**Fig. 2H**; **new Fig. 2I** in the revised manuscript) resulted in larger primary tumors compared to CMTR2 KO, CMTR2 loss produced a markedly stronger effect on spontaneous metastasis (**new Figs. 2m, 2n** in the revised manuscript). This apparent discrepancy highlights an important and well-established concept in cancer biology: primary tumor growth and metastatic potential are not necessarily coupled. Indeed, clinical studies in breast cancer have shown that the relationship between tumor size and distant metastasis is not strictly linear, as metastatic dissemination is largely governed by tumor-intrinsic biological programs rather than tumor burden alone. Consistent with this, loss of both NF2 and CMTR2 enhanced lung metastasis in our model, although CMTR2 deficiency elicited a more pronounced metastatic phenotype.

Mechanistically, this divergence is supported by the distinct biological functions of these genes. NF2 is a core regulator of the Hippo pathway and primarily controls cell proliferation and tissue growth, which may explain its stronger impact on primary tumor expansion. In contrast, CMTR2 is a key RNA cap methyltransferase that regulates mRNA processing, stability, and translation. Its loss is therefore likely to induce broader transcriptomic and translational reprogramming, potentially affecting pathways involved in epithelial-to-mesenchymal transition, invasion, intravasation, and tumor–microenvironment interactions, thereby preferentially promoting metastatic competence. Importantly, both NF2 and CMTR2 are significantly downregulated in TNBC patient datasets (UALCAN; **Fig. 4b**), supporting their roles as clinically relevant suppressors of tumor progression. Together, these findings suggest that NF2 predominantly constrains primary tumor growth, whereas CMTR2 plays a more dominant role in limiting metastatic dissemination, underscoring the functional heterogeneity of metastasis suppressor genes.

This point has now been discussed and added to the discussion section, page 15: “Notably, our data reveal a dissociation between primary tumor growth and metastatic potential. While NF2 loss preferentially enhanced tumor expansion, CMTR2 deficiency more strongly promoted metastatic dissemination, consistent with evidence that metastatic competence is not dictated by tumor size alone. This divergence likely reflects distinct gene functions, with NF2 primarily regulating proliferation via Hippo signaling, whereas CMTR2 loss may drive broader transcriptomic reprogramming that facilitates invasion and colonization. These findings underscore the functional heterogeneity of metastasis suppressor genes and the need to distinguish regulators of tumor growth from those governing metastatic progression”.

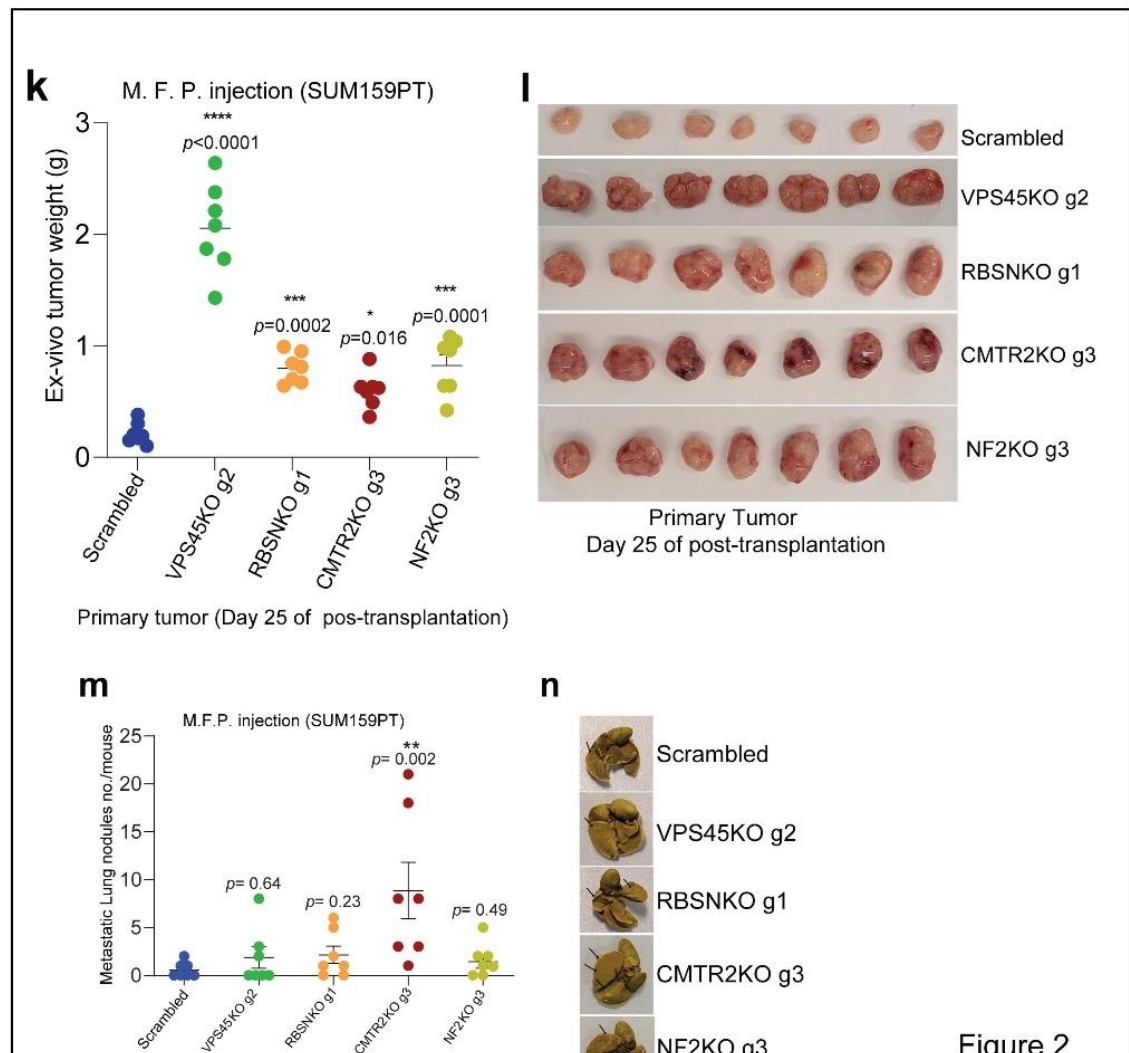


Figure 2

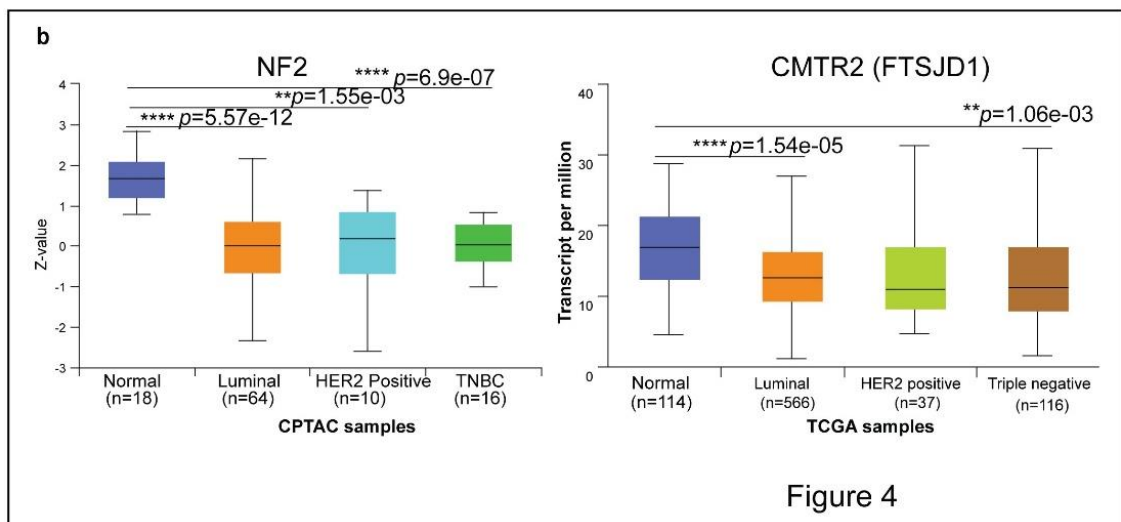


Figure 4

3. The authors report a correlation between the four MSGs and patients' relapse-free survival and overall survival (Figure 3D). It would be valuable to confirm these findings in an animal model.

We thank the reviewer for this insightful suggestion. We agree that linking MSG expression to survival outcomes in vivo would provide an additional layer of validation. However, the primary objective of this study is to functionally interrogate metastasis suppressors in the context of tumor dissemination and metastatic burden, rather than overall survival endpoints, which can be influenced by multiple systemic variables beyond metastatic progression alone. Importantly, several of the identified MSGs are known to play essential roles in development, which complicates the interpretation of survival-based studies in vivo. For example, constitutive loss of CMTR2 results in embryonic lethality around mid-gestation (E10.5–E11.5)¹, and VPS45 knockout is similarly incompatible with embryonic viability². Likewise, homozygous loss of NF2 leads to early embryonic lethality, while heterozygous animals develop highly metastatic tumors³. These findings highlight that survival outcomes in whole-animal models may reflect fundamental developmental requirements rather than tumor-specific functions, thereby confounding their interpretation in the context of metastasis suppression. Furthermore, our study integrates orthogonal lines of evidence by combining patient-derived survival associations with direct in vivo functional validation. We demonstrate that CRISPR-mediated knockout or activation of these genes significantly alters metastatic progression, providing mechanistic support for the clinical correlations observed in human datasets and establishing a causal role for these MSGs in regulating metastasis. To further clarify the conceptual and experimental framework of our study, we have included a new schematic summary (**Fig. 9**) in the revised manuscript, highlighting how the identified MSGs functionally contribute to metastatic progression. We agree that dedicated studies evaluating survival outcomes in preclinical models would be of interest and represent a valuable direction for future investigation.

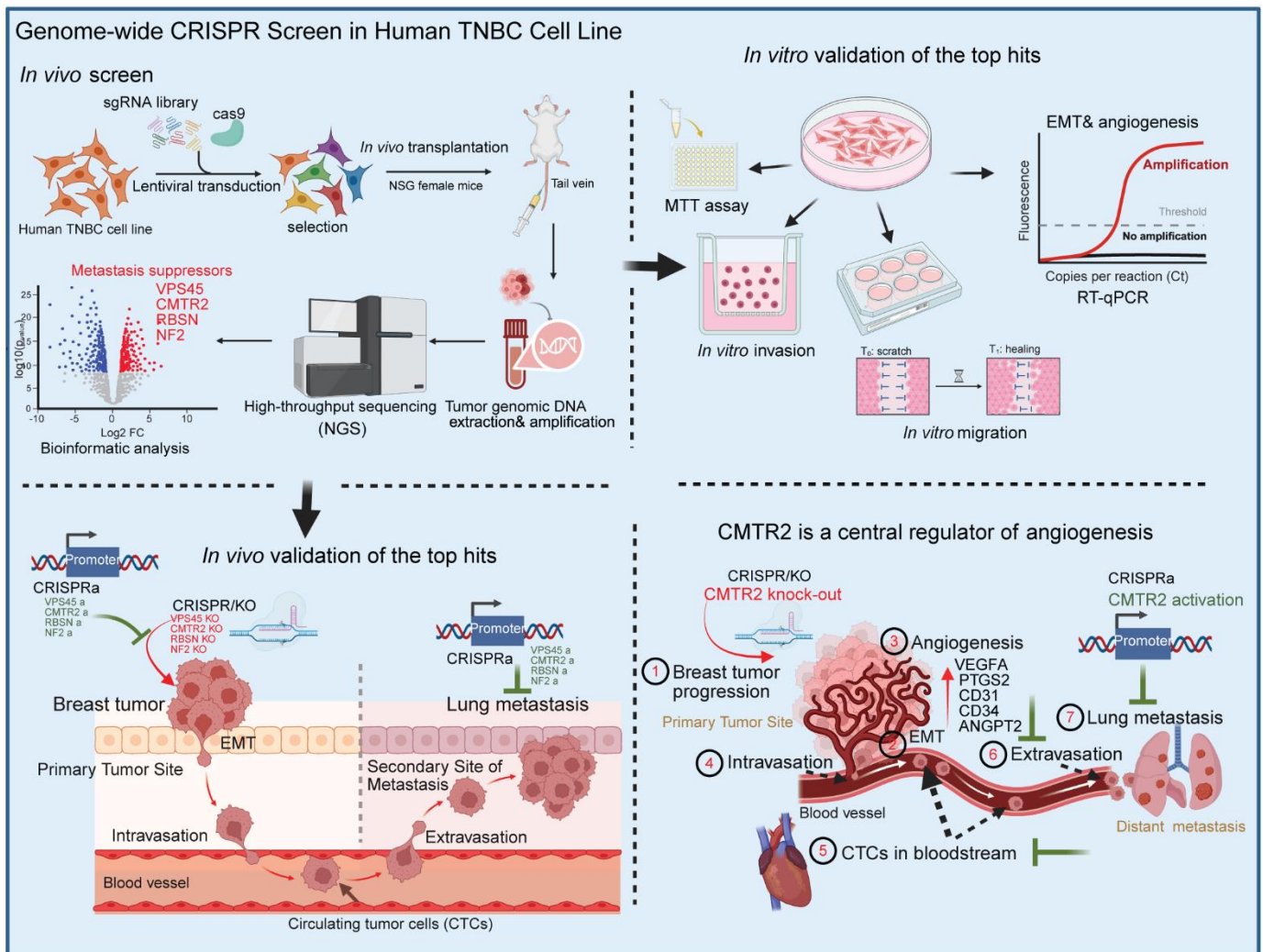
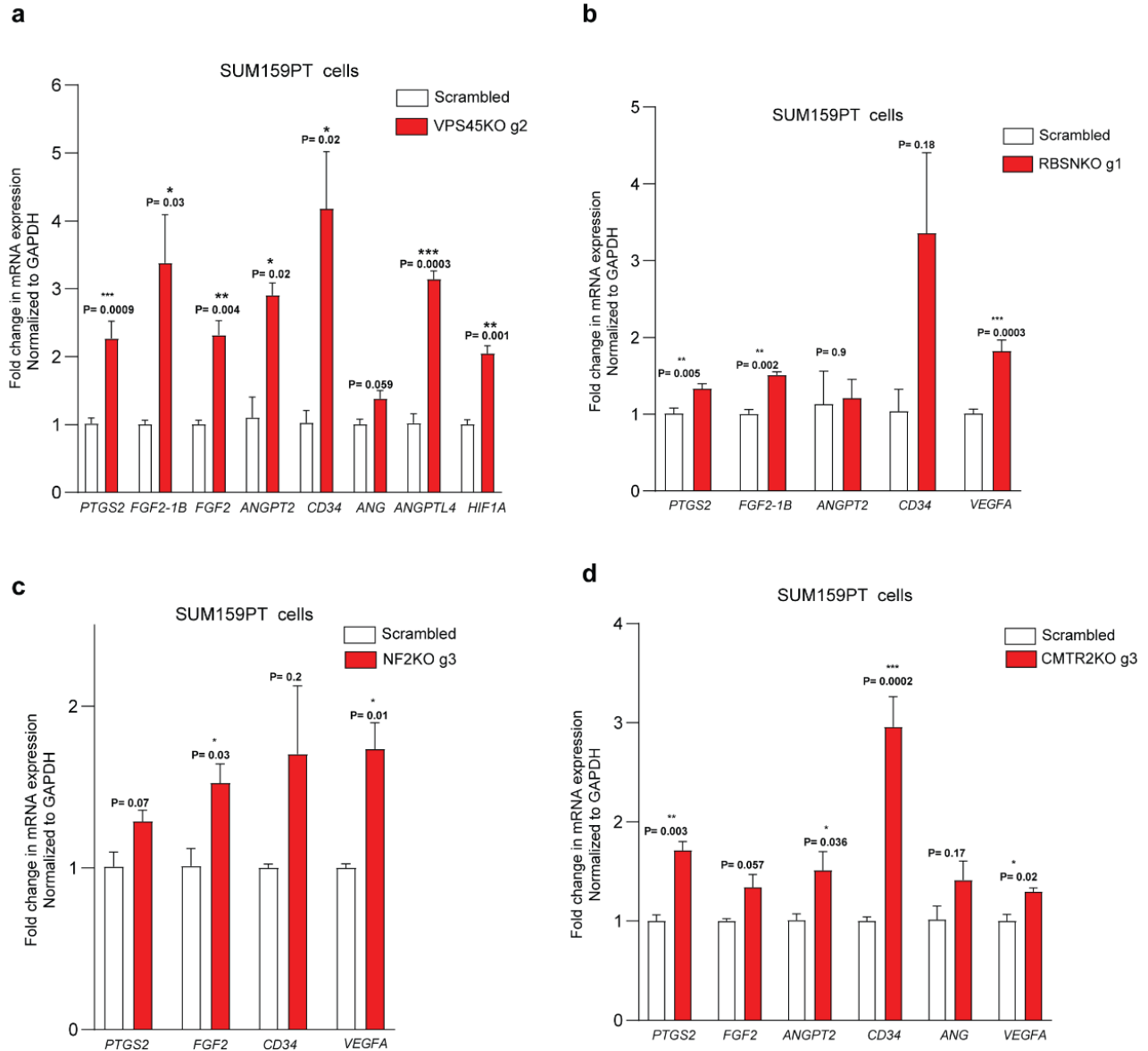


Fig. 9. Schematic summary of the identification and validation workflow of lung metastasis suppressors in TNBC. An *in vivo* human genome-wide CRISPR knockout screen was performed in human TNBC cells to identify regulators of lung metastatic colonization using NSG female mice. The enriched sgRNAs in lung metastases were analyzed to determine top candidate metastasis suppressors. Functional validation of selected candidates (VPS45, CMTR2, RBSN, and NF2) was conducted using *in vitro* assays, including viability, RT-qPCR, invasion, and wound-healing assays, as well as *in vivo* models through orthotopic and intravenous tail vein transplantation. Both loss-of-function by CRISPR knock out (CRISPR/KO) and gain-of-function by CRISPR activation (CRISPRa) approaches confirmed the anti-metastatic roles of the top candidates, thereby validating the CRISPR lung metastasis screen. Besides revealing CMTR2 as a central regulator of angiogenesis *in vivo*.

4. It is important to present the expression levels of key growth factors, such as VEGF and FGF, to substantiate the claim that MSGs regulate angiogenesis in the cell culture study.

We thank the reviewer for this constructive suggestion. While our original submission provided evidence of angiogenic regulation through markers such as *PLAU*, *KAT2B*, and *CTGF*, we recognize the importance of validating these findings with additional canonical growth factors. To directly address this, we expanded our analysis in the SUM159 model using RT-qPCR. This new data reveals a robust and coordinated upregulation of multiple key angiogenesis-associated genes, including *VEGFA*, *FGF2*, the *FGF2*-1B isoform, *ANGPT2*, *ANGPTL4*, *ANG*, *CD34*, *HIF1A*, and *PTGS2* (**New Supplementary Fig. 6**); unpaired Student's t-test, $p < 0.05$). The induction of *VEGFA*, *ANGPT2*, and *CD34* is particularly significant, as these genes typically exhibit low basal expression in standard in vitro culture lacking hypoxic or stromal cues. Their upregulation indicates the activation of a specific angiogenic transcriptional program rather than baseline variability. Furthermore, the concurrent increase in the master regulator *HIF1A* and its downstream effector *PTGS2* provides a mechanistically coherent link between MSG perturbation and the resulting pro-angiogenic phenotype.

Manuscript Changes: We have added **New Supplementary Fig. 6** and updated the Results section on Page 10: *"Building upon our initial findings of increased PLAU and CTGF expression, we expanded our analysis to include canonical angiogenic regulators in the SUM159 model. We observed a robust induction of several key factors typically expressed at low basal levels in vitro, most notably VEGFA, ANGPT2, and CD34 (Supplementary Fig. 6). Furthermore, the concurrent upregulation of HIF1A and its downstream effector PTGS2 suggests that MSG perturbation activates a mechanistically coherent angiogenic transcriptional programs. Additionally, upregulation of ANG, FGF2, and ANGPTL4 also were detected, suggesting that VPS45, RBSN, CMTR2, and NF2 may not only regulate the EMT process but also promote angiogenesis, thereby amplifying the metastatic potential of TNBC cells".*



Supplementary Fig. 6 Loss of VPS45, RBSN, CMTR2, and NF2 induces expression of angiogenic-associated genes. Real-time qPCR analysis of angiogenesis-related genes expression using total RNA purified from transduced VPS45, RBSN, NF2, and CMTR2 SUM159KO cells and normalized to GAPDH mRNA and expressed relative to non-targeting (Scrambled) control. Depletion of the 4 targets resulted in increased expression of multiple angiogenesis-associated genes. Data are presented as mean $\pm$ SEM ($n = 3$ replicates). Statistical significance was determined using Student's t-test (two-sided and unpaired) analysis; * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

5. Were any observations made regarding the effect of these TSGs on brain metastasis?

No brain metastases were observed in our study. This is consistent with the known organ tropism of the models used: both MDA-MB-231 and SUM159, including their CRISPR-modified derivatives, preferentially colonize the lungs following systemic dissemination in NSG mice, while spontaneous brain metastasis remains rare under these conditions. Robust central nervous system (CNS) colonization typically requires specialized experimental approaches, such as brain-tropic derivatives (e.g., MDA-MB-231-BR) and intracardiac injection to bypass first-pass organ filtering and homing constraints. Therefore, the absence of brain lesions reflects established model-specific dissemination patterns rather than an absence of metastatic capability or gene-specific effects. Importantly, this does not impact the interpretation of our findings in lung metastasis. Evaluation of these tumor suppressor genes in CNS metastasis using dedicated brain metastasis models would represent a logical and important extension of this work.

6. Statistical Analysis: The authors used two-tailed unpaired t-tests. However, some experiments compared multiple samples to a control (Scrambled). For comparisons involving three or more group means, ANOVA (Analysis of Variance) is more appropriate than performing multiple t-tests.

We thank the reviewer for this important suggestion. We agree that for comparisons involving more than two groups, an analysis of variance (ANOVA) framework is generally appropriate. However, inspection of our data revealed deviations from normality, particularly for metastatic burden measurements (lung nodule counts), which are inherently skewed and do not meet the assumptions of parametric tests. Accordingly, for multi-group comparisons, we employed the Mann–Whitney *U* test, which does not assume normal distribution. These approaches are widely used for in vivo metastasis studies where count-based or non-Gaussian data are common.

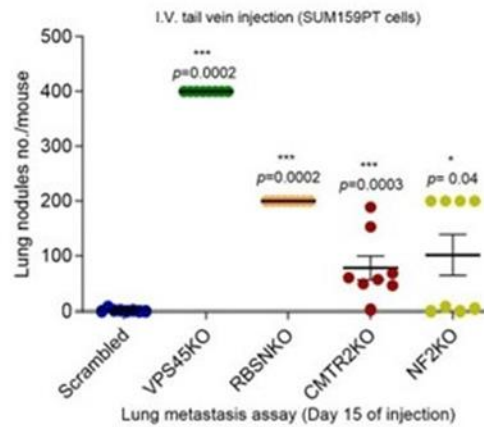
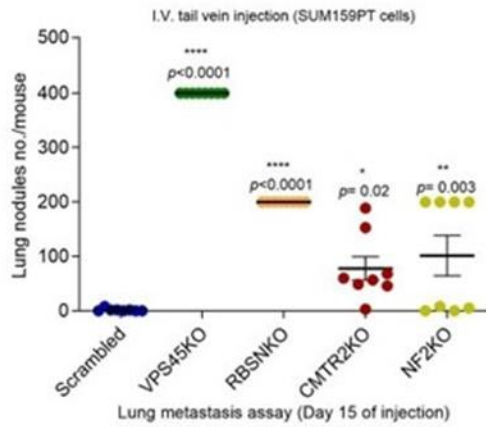
Importantly, we also reanalyzed the relevant datasets using parametric one-way ANOVA with Mann–Whitney *U* test, and this yielded consistent results in terms of both statistical significance and overall interpretation, supporting the robustness of our findings. The statistical methodology and rationale have now been clarified in the Methods section.

As shown below, for the analysis of lung nodule counts, applying one-way ANOVA did not alter the significant effect of CMTR2KO on spontaneous metastasis. However, due to the non-normal distribution among groups, we used the Mann–Whitney *U* test. Since non-normal distribution was observed for both lung nodule counts and circulating tumor cell counts, we retained the Mann–Whitney *U* test for statistical analysis.

Ordinary One-way ANOVA analysis



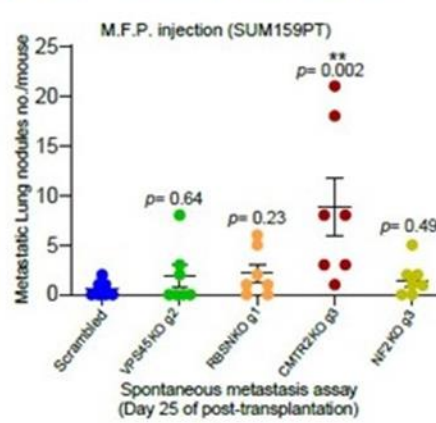
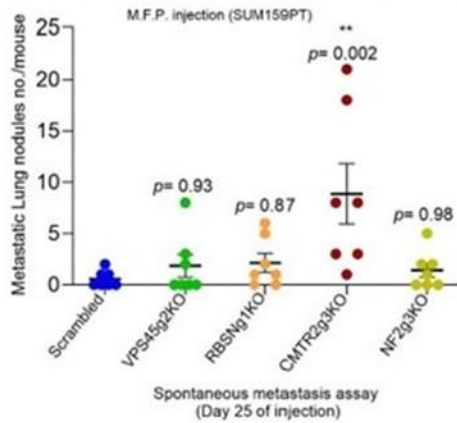
Mann-Whitney *U* test analysis



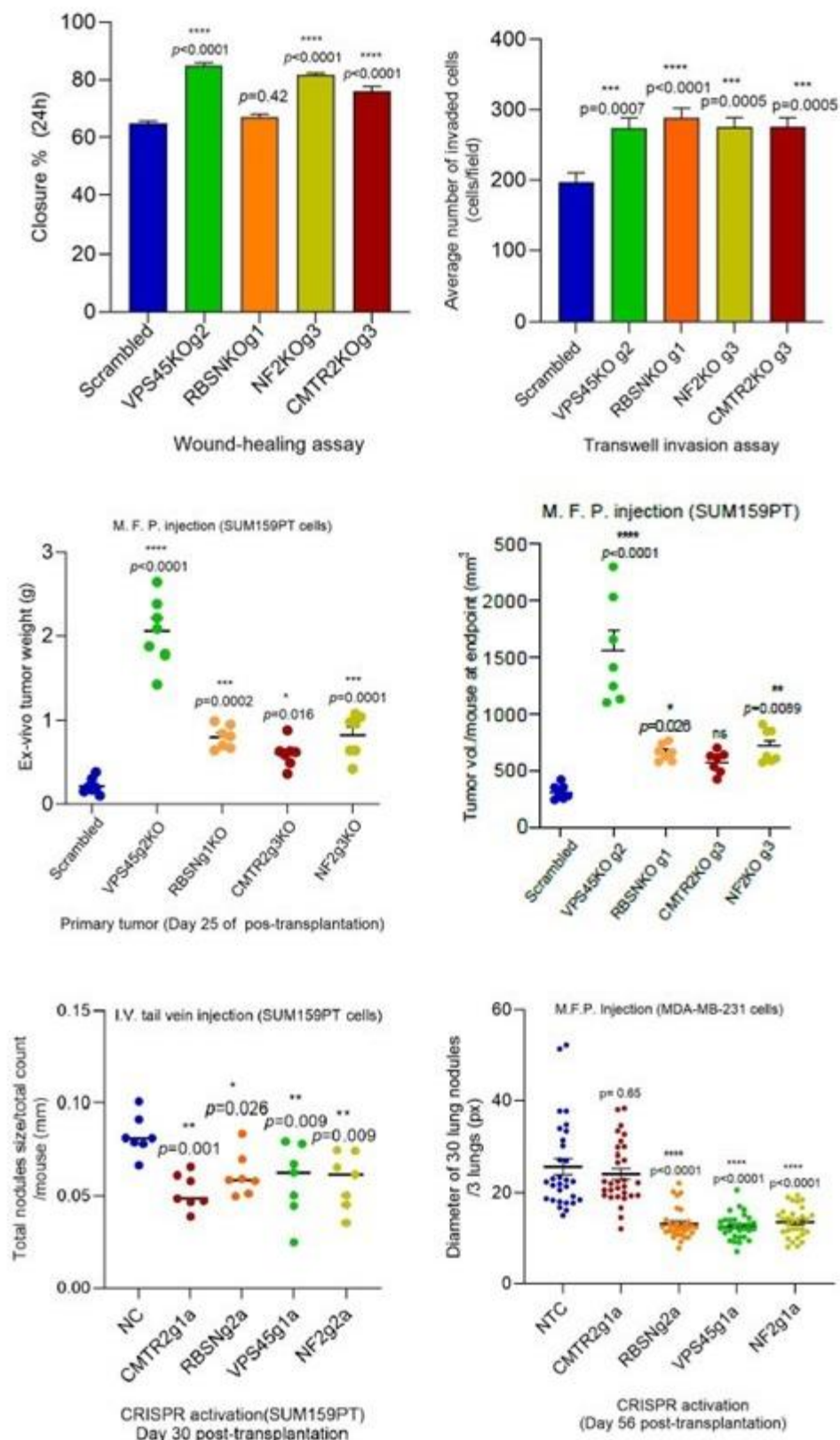
Ordinary One-way ANOVA analysis



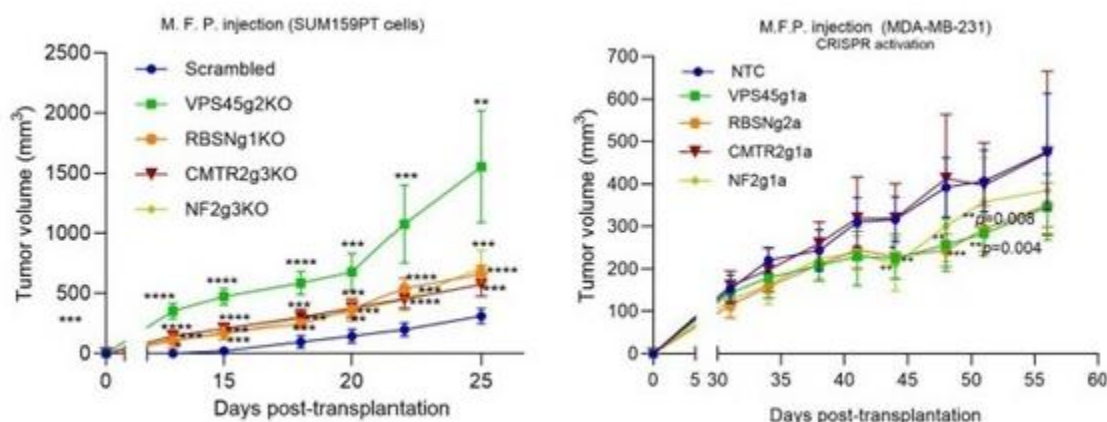
Mann-Whitney *U* test analysis



For all data that show normal distribution Ordinary one-way ANOVA analysis was used, as shown below.



Repeated measures ANOVA was used for tumor volume analysis because tumor growth was measured longitudinally in the same animals across multiple time points which allows more accurate assessment of growth differences between groups over time.



Minor comments

Multiple places TNBC is written as TBNC. Please correct

We sincerely thank the reviewer for identifying this error and apologize for the oversight. The abbreviation has been corrected throughout the revised manuscript.

Most of the results highlight the roles of four TSGs, while only a few experiments specifically examine the effect of CMTR2 alone. It would be more appropriate to revise the title to reflect the involvement of all four TSGs.

We thank the reviewer for this insightful suggestion. We agree that the title should accurately reflect the scope of the study and not be centered solely on CMTR2. Accordingly, we have revised the title to more broadly reflect the identification of multiple metastasis suppressor genes rather than emphasizing a single candidate.

Revised title: *In Vivo CRISPR Screening Identifies Metastasis Suppressors in Triple-Negative Breast Cancer*

- 1 Yermalovich, A. V. *et al.* An essential role for Cmtr2 in mammalian embryonic development. *Dev Biol* **516**, 47-58 (2024). <https://doi.org/10.1016/j.ydbio.2024.07.019>
- 2 Frey, L. *et al.* Mammalian VPS45 orchestrates trafficking through the endosomal system. *Blood* **137**, 1932-1944 (2021). <https://doi.org/10.1182/blood.2020006871>
- 3 McClatchey, A. I. *et al.* Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. *Genes Dev* **12**, 1121-1133 (1998). <https://doi.org/10.1101/gad.12.8.1121>

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors were exceptionally responsive to the issues raised by all three reviewers, including completion of extensive new experiments. All of my concerns are satisfied. I am highly supportive of publication. I congratulate the authors on a rigorously conducted and fascinating study.

Reviewer #2 (Remarks to the Author):

The authors have responded thoroughly to my comments and have significantly strengthened the manuscript. I therefore support its publication.

We were pleased to see that all reviewers were satisfied with the extent and rigor of our revisions and had no further comments.

Sincerely,

Dr. Jean-Jacques Lebrun