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USP8 promotes cancer progression and extracellular vesicle-mediated CD8+ T cell exhaustion by deubiquitinating the TGF-β receptor TβRII

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Dear Prof. Zhou,

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find that the analysis interesting and suitable for consideration here. They raise a number of different issues that I would like to ask you to resolve in a revised version. I think it would be helpful to discuss the revisions further and I am happy to do it via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discuss your revisions further with you.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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Referee #1:

In the manuscript, Xie et al suggest that targeting USP8 may inhibit cancer metastasis and prevent extracellular-vesicle TBRII-induced exhaustion of CD8+T cells. The manuscript described the role of TGFB-signaling in tumor progression and immune evasion. In particular, the authors suggest that USP8 is a deubiquitnase that modulates TBRII stability. This translate in intrinsic increase in TGFB cancer cell signaling but also in extracellular vesicles loaded with such receptor. The authors suggest that this vesicles in turn impact CD8+ T cells exhausted subsequently returning them anti-tumor activity. Overall this is an interesting study that aims to couple two TGFb dependent effects caused by a unique alteration in the cancer cells. This is a well-executed manuscript, that provides relevant information albeit there are some conceptual and technical hurdles that need to deal with.

The manuscript systematically builds on the concept of metastasis. This is catchy but inaccurate in this occasion. USP8 effects are very clear at the primary site and at the distant sites. In this case driven by the same mechanism of action. It is well established in the field that the larger a tumor is the higher the risk of metastasis, yet that does not mean that a molecular alteration of the first drives the second. In this regard, unless differences in metastasis are observed in experiments with size-match tumors the title and the text should be tuned down. For example, referring to "... breast cancer progression and prevents...".

The associations described in figure 1c are referred to all BCa. Does it improved if focused on basal-like? Similarly, does USP8 expression correlate with TGFb response signature?

In figure 1i and subsequently in BLI images, the plots y axis reflects a very narrow range. If there is no lesion, background signal should be low and a wider range used to highlight the differences. A log transformation of the values may help. If no metastasis the mice should not be scored in the total photon flux curve and the Kaplan-Maier suffice to highlight it.

MCF10A cells require Ras and ERBB2 to lose TGFbeta cytostatic response (Chen et al PNAS 2001). In addition, this model is thought to be prone to senescence (Arnal-estapé Cancer research 2010). In this context, it does not seem the appropriate model to test metastatic capacity. In addition, why in figure 2i, left panel, there is a flag expressed construct in the shRNA stable MDA-231 cells? Was Flag USP8 expressed in this control?

Similarly, why HaCaT cells were chosen. This are keratinocytes that maintain a good TGFb cytostatic response, but keratinocytes and this manuscript was about breast cancer. The supposed EMT process should be score in the latter cell type.

In figure 3, IHC analyses are depicted. It is unclear whether USP8 antibody was validated. USP expressing and depleted cell pellets should be used to show antibody specificity. Similarly, references and protocols for the TbRII and P-Smad antibodies should be provided or alternatively validated with loss of expression cell pellets.
How extracellular vesicles compared with the basal TGFbeta activity? Are these two activities coupled? Is all the TGFb effect seen in clinical sets due to USP8?

One wonders what would happen when inhibiting USP8 in vivo in mice with established lesions (size matched) and then treated as opposed to what is shown in figure 6. AS depicted, pretreated mouse represent a non clinical setting. Further, how is the TGFb signaling pathway in this cells (in the epithelial compartment)?

Finally, the last figure is perceived as out of the blue. The data is interesting but is not explained what is the rationale of the combination with PDL1, why focusing on T cells and not fibroblasts for example. In addition, what is the effect of PDL1 and USP8i in the absence of the vesicles formation? How does this compare with the use of a TGFb inhibitor with anti PDL1 (i.e. galunisertib)

Referee #2:

The manuscript by Xie et al., investigates the role that ubiquitin-specific peptidase 8 (USP8) plays in stabilizing the TGF-beta type II receptor (TβRII), which enhances TGFβ signaling within cancer cells and promotes an epithelial-to-mesenchymal transition (EMT) and breast cancer cell migration/invasion. In addition, USP8 increases the amount of TβRII that is packages into extracellular vesicles (EVs), which can suppress T cell activation and anti-tumor immunity. Interfering with USP8 function impairs TGFβ signaling and diminishes the number of TβRII+ EVs, which leads to increased T cell mediated clearance of breast cancer cells.

The authors have built a compelling story that is supported by a significant amount of biochemical, cell biology and in vivo tumor growth and metastasis data. When possible, the authors have examined correlations between USP8 levels and the degree of TGFβ signaling using IHC analyses of human clinical samples and publicly available gene expression data from breast cancer patients. The conclusions drawn are well-supported by the data and will be of interest to the broader cancer community.

Specific Points:
The authors suggest that stable knockdown of USP8 diminishes the stem cell characteristics of breast cancer cells - have specific stem cell markers been assessed in the various breast cancer models that do or do not express USP8?

The authors suggest that USP8 levels are correlated with mesenchymal markers and that USP8 is required for the TGFβ induced expression of EMT markers in certain cell models. Is loss of USP8 sufficient to induce a mesenchymal-to-epithelial transition in basal breast cancer cells?

It appears that endogenous USP8 expression in MDA-MB-231 cell is quite low (Fig. S1b) relative to other basal breast cancer models. It also seems that some of the knockdown studies performed in the MDA-MB-231 model were done in cells engineered to overexpress USP8 (Figure 2i). Is the endogenous level of USP8 in MDA-MB-231 cells sufficient to have an impact on endogenous TGFβ signaling? Is the data shown Fig. 1h a knockdown of endogenous USP8 in MDA-MB-231 cells? It would help for the authors to clarify this point. Also, have USP8 knockdowns been performed in other basal breast cancer models that express higher levels of USP8 (MDA-MB-436) been performed?

The authors suggest that overexpression of USP8 is correlated with the machinery that produces EVs. They also show that high USP8 levels are correlated with an increase in the number of TβRII+ EVs. Given that loss of USP8 results in a 25% decrease in overall EV secretion but a dramatic reduction in the formation of TβRII+ EVs, do the authors believe that USP8, through removal of ubiquitin from TβRII, results in a greater receptor pool that can be packaged into EVs, or that USP8 directly binds to TβRII and actively chaperones TβRII into EVs?

The inhibitor caused a 25% reduction in total vesiculation, which was comparable to what was seen in the USP8 knockdown cells. Does the inhibitor show any effect on total vesiculation in cells with a USP8 knockdown - these experiments would reveal any potential off target effects of the UPS8i.

Can the authors revisit the USP8 staining in the breast tumor TMA to see if high USP8 levels correlate with fewer infiltrating CD8+/GZMB+ T cells (immune cold) relative to tumors that express lower levels of USP8 (immune hot)? Or can the gene expression data be analyzed via a CYBERSORT approach to estimate immune cell populations (CD8+ T cells) in USP8 high or low breast tumors?

The authors suggest two possible roles for USP8 in enhancing the ability of breast cancer cells to become more metastatic. The first is through elevated levels of the TβRII, which enhances TGFβ signaling and increased stemness, migration/invasion and metastasis (tumor intrinsic). This clearly occurs in xenograft models where T cell anti-tumor immunity is not a factor (MDA-MB-231). The second is through the potential action of TβRII+ EVs on the suppression of T cell mediated anti-tumor immunity. Do the authors have a sense of what is the predominate mechanism at play in the syngeneic model (4T1). If 4T1 cells are injected into an immunocompromised background, does the USP8i still impair tumor growth (as seen in Balb/c mice - Fig. 7a, b)? If TβRII+ EVs are harvested and injected into Balb/c mice bearing 4T1 breast tumors - does this increase tumor growth and metastasis relative to TβRII- EVs?

Referee #3:

Xie et al. uncover a deubiquitinating enzyme (DUB) responsible for stabilization of TBRII at the cell surface, thereby influencing EMT and cancer stemness properties, as well as progression of breast cancer to metastatic disease in vivo. High levels of this DUB, USP8, are found in patient tumor samples compared to normal tissue and correlate with poor metastasis free survival. Additionally, they find that reducing the levels of TBRII at the surface influences the quantity of TBRII in circulating extracellular vesicles (EVs), which correlates with reduced primary and metastatic tumor burden and alters expression of several proteins in T cells. A USP8 inhibitor, both alone and synergistically with paclitaxel and anti-PD-L1 therapy, reduces tumor burden and increases active CD8+ T cells. Overall, this is a very thorough and interesting paper with a few conclusions that could be strengthened.

Major concerns:

Is it the increased EV-TBRII or increased TBRII in primary/circulating/metastatic tumor cells that is responsible for changes in disease progression? Much of the evidence presented is correlative and not causative for EV involvement. The manuscript generally does not directly state that the EVs are responsible for the outcomes observed, however the title of the manuscript does make this conclusion. To provide direct evidence, could the authors co-culture TBRII+EVs with cancer cells and see a change in migration/invasion for example or with T cells to look for activation and proliferation changes? These experiments of course cut out the complex in vivo environment. In the materials and methods section the authors mention an "EVs education experiment..." on page 25. Have the authors attempted to inject altered EVs into mice to look for changes in a syngeneic manner? This would be a powerful experiment to include to address the direct role of EVs and support the conclusions drawn in the title.

USP8, of course, has target proteins other than TBRII that likely are contributing to the observed outcomes. In the literature, it is demonstrated that EGFR and HIF1a are both validated targets. Can the author's perform experiments that either re-express or knockdown TBRII in USP8 modulated cells to determine the relative impact of TBRII on the overall effect of USP8? In vivo tumorigenic and metastatic studies would be most convincing.

Minor concerns:

• In Fig. 1d, what diameter cut off is used to count mammospheres?
• Were any metastatic lesions observed in the in vivo experiment depicted in Fig. 1e?
• What method is used in Fig. 2h? I can't find where it is clearly described whether this is qPCR or luciferase data.
• What is Flag tagged in Fig. 2i left panel?
• Add another mesenchymal marker such as vimentin to the immunofluorescence panel in 2j.
• On page 10 an experiment was described with flag-tagged TBRII and HA-ubiquitin but I cannot find this experiment in the figures.
• Was ubiquitination of TBRI by USP8 also investigated and ruled out?
• Please add legends to 4f panel 2 and 4g.
• 'Gene signatures representing vesicle formation and cancer metastasis' were mentioned in the text describing Figure 5a, however metastasis is not shown. Could this be added/ corrected?
• When was inhibitor treatment initiated in Fig. 6k?
• On page 16, TBRII mRNA levels are referenced but the figure is not shown.
• Inconsistent spelling (e.g. tumour vs tumor) and other minor typos throughout (e.g. 'bresat' in Figure 6i).

We thank all referees for their constructive criticisms, suggested clarifications and textual modifications. All these efforts greatly helped us and strongly improved this study.

Referee #1:

In the manuscript, Xie et al suggest that targeting USP8 may inhibit cancer metastasis and prevent extracellular-vesicle TBRII-induced exhaustion of CD8+T cells. The manuscript described the role of TGFB-signaling in tumor progression and immune evasion. In particular, the authors suggest that USP8 is a deubiquitnase that modulates TBRII stability. This translate in intrinsic increase in TGFB cancer cell signaling but also in extracellular vesicles loaded with such receptor. The authors suggest that this vesicles in turn impact CD8+ T cells exhausted subsequently returning them anti-tumor activity. Overall this is an interesting study that aims to couple two TGFb dependent effects caused by a unique alteration in the cancer cells. This is a well-executed manuscript, that provides relevant information albeit there are some conceptual and technical hurdles that need to deal with.

The manuscript systematically builds on the concept of metastasis. This is catchy but inaccurate in this occasion. USP8 effects are very clear at the primary site and at the distant sites. In this case driven by the same mechanism of action. It is well established in the field that the larger a tumor is the higher the risk of metastasis, yet that does not mean that a molecular alteration of the first drives the second. In this regard, unless differences in metastasis are observed in experiments with size-match tumors the title and the text should be tuned down. For example, referring to "... breast cancer progression and prevents...".

Response: We agree with this reviewer and thus turned down the related descriptions in title: "Targeting USP8 inhibits Cancer Progression and Prevents Extracellular-Vesicle TβRII-Induced Exhaustion of CD8$^+$ T cells" and also made proper changes in main text (highlighted in Page 20). Many thanks for suggestion.

The associations described in figure 1c are referred to all BCa. Does it improved if focused on basal-like? Similarly, does USP8 expression correlate with TGFb response signature?

Response: Although USP8 expression is significant higher in the basal-like than that in the normal control (*Figure for reviewer only 1*), the result is similar to Figure 1c. This might due to a broad range of USP8 expression which also indicates that USP8 might be regulated by multiple signal inputs that were not yet elucidated.

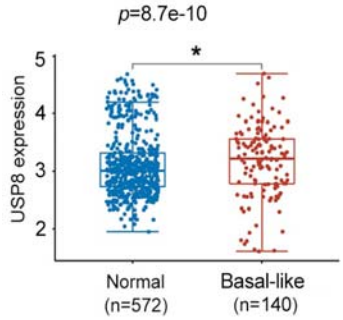

***Figure for reviewer only 1*: The expression distribution of USP8 gene in Basal-like tumor tissues**

(n = 140) and normal tissues (n = 572), where the horizontal axis represents different groups of samples, the vertical axis represents the gene expression distribution.

Yes, the USP8 expression positively correlates with TGF-β response signature: we showed that USP8 expression positively correlates with the increased expression of several TGF-β target genes involved in cancer cell invasion and metastasis, including *Snai1* and *Snai2* (*Slug*) (Fig. 2c and Supplementary Fig. 2a). In breast cancer tissue microarray analysis, USP8 expression also positively correlates with p-SMAD2 and TβRII expression (Fig. 3m, 3n and Supplementary Fig. 3d, 3f).

In figure 1i and subsequently in BLI images, the plots y axis reflects a very narrow range. If there is no lesion, background signal should be low and a wider range used to highlight the differences. A log transformation of the values may help. If no metastasis the mice should not be scored in the total photon flux curve and the Kaplan-Maier suffice to highlight it.
Response: In response to reviewer, we modified Figure 1i, many thanks for suggestion.

MCF10A cells require Ras and ERBB2 to lose TGFbeta cytostatic response (Chen et al PNAS 2001). In addition, this model is thought to be prone to senescence (Arnal-estapé Cancer research 2010). In this context, it does not seem the appropriate model to test metastatic capacity. In addition, why in figure 2i, left panel, there is a flag expressed construct in the shRNA stable MDA-231 cells? Was Flag USP8 expressed in this control?
Response: During EMT, cells do not necessarily exist in 'pure' epithelial or mesenchymal states. There are cells with mixed (or hybrid) features of the two, which are termed as the intermediate cell states (ICSs). While the exact functions of ICS remain elusive, together with EMT it appears to play important roles in pathological processes such as cancer metastasis. As this reviewer has pointed out, MCF10A cells do require Ras to escape the growth inhibition caused by TGF-β. Although MCF10A human breast epithelial cell line maintain features of normal breast epithelial cells, its derivative, RAS-transformed MCF10A cells (MCF10A-RAS, also named as MII) show ICSs or complete EMT phenotype upon TGF-β activation. In figure 2d-e, MCF10A-RAS cell were used for mammoasphere formation *in vitro* and xenografts *in vivo*, and they were also used in similar assays of our previous studies [1]. Moreover, we also confirmed the pro-metastatic potential of USP8 by using well-defined MDA-MB-231 and 4T1 cell models (Figure 4&5).

The "Flag" in Figure 2i should be "USP8". We corrected this mistake. Many thanks for pointing out this typo for us.

Similarly, why HaCaT cells were chosen. This are keratinocytes that maintain a good TGFb cytostatic response, but keratinocytes and this manuscript was about breast cancer. The supposed EMT process should be score in the latter cell type.
Response: As this reviewer has pointed out, HaCaT remains as the most sensitive cell type to TGF-β-induced cytostatic response and EMT. We thus used this model to artificially over-express USP8, in which we nicely demonstrated that the gain function of USP8-wt, but not USP8-cs, could mimic TGF-β function in driving EMT (Figure 2j and

Supplementary Figure 2c). This cell type was also previously employed in similar situations [1-3]. As requested by this reviewer, we also used MCF10AR-RAS (MII) cells to examine EMT (Figure 2k).

In figure 3, IHC analyses are depicted. It is unclear whether USP8 antibody was validated. USP expressing and depleted cell pellets should be used to show antibody specificity. Similarly, references and protocols for the TbRII and P-Smad antibodies should be provided or alternatively validated with loss of expression cell pellets.

Response: USP8 antibody specificity were actually validated by USP8 expressing and depleted cell pellets (see the input blots of USP8 in Fig 2i, 2k and Fig 3h, 3j). TβRII and P-SMAD2 antibodies were validated below (*Figure for reviewer only 2*). Many thanks for suggestion.

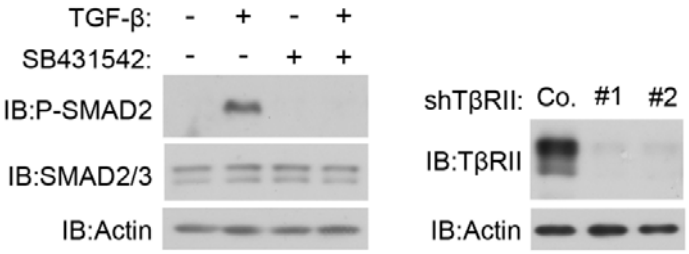

Figure for reviewer only 2: **Immunoblot analysis of total cell lysates derived from MDA-MB-231 cells stimulated with TGF-β (2.5 ng/ml 1 h) without or with the pre-treatment of SB431542 (10 μM 2.5 h) (left). Immunoblot analysis of total cell lysates derived from control and TβRII stably depleted (#1 and #2 shRNA) MDA-MB-231 cells (right).**

How extracellular vesicles compared with the basal TGFbeta activity? Are these two activities coupled? Is all the TGFb effect seen in clinical sets due to USP8?

Response: USP8 deubiquitinates thus stabilizes TβRII on the plasma membrane (Figure 3). Gain of USP8 function enhances secretion of TβRII$^+$ crEVs (Figure 4). USP8 loss of function by either shRNA-mediated knockdown or the treatment with specific inhibitor all lead to reduced levels of TβRII protein and EV secretion (Figure 5&6).

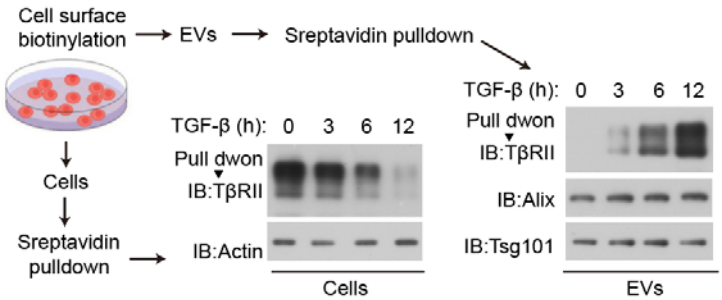

Figure for reviewer only 3: **Schematic diagram of biotin-labeling assay (left) and immunoblot analysis (right) measuring TβRII levels on the cell membrane and secreted EVs.**

In response to reviewer, we labeled the cell surface TβRII with biotin and observed the biotin-TβRII in EVs (*Figure for reviewer only 3*). This result indicates that EV-TβRII is

derived from the surface of donor cells. Therefore, USP8 inhibition can reverse basal TGF-β activity by repressing TβRII protein stability in donor cells and meanwhile reduce secretion of EV-TβRII, showing that those two events are indeed coupled.

As we had investigated, the pro-malignant effects of TGF-β in breast tumors can be mediated by USP8 via enhancing TβRII stability and the downstream canonical SMAD activation. We can not conclude that all the clinical TGF-β effect due to USP8 as this must require rather broad and very comprehensive investigations.

One wonders what would happen when inhibiting USP8 in vivo in mice with established lesions (size matched) and then treated as opposed to what is shown in figure 6. AS depicted, pretreated mouse represent a non clinical setting. Further, how is the TGFb signaling pathway? in this cells (in the epithelial compartment

Response: In response to reviewer, we performed a new batch of mice experiment in which above mentioned setting (close to clinics) were established for analysis (see revised Supplementary Figure. 6c-f): MDA-MB-231-Luc cells (1×10^5 per mouse) were firstly intracardially injected into nude mice (n = 5 for each group). After 3 weeks, USP8 inhibitor (1 mg/kg) was injected via intraperitoneal injection every the other day for three weeks. As shown, the tumor development was suppressed in mice with established lesions (size matched) upon treatment with USP8 inhibitor; TGF-β/SMAD signaling was inhibited in tumors. Related descriptions were highlighted in Page 18. Many thanks for this suggestion.

Finally, the last figure is perceived as out of the blue. The data is interesting but is not explained what is the rationale of the combination with PDL1, why focusing on T cells and not fibroblasts for example. In addition, what is the effect of PDL1 and USP8i in the absence of the vesicles formation? How does this compare with the use of a TGFb inhibitor with anti PDL1 (i.e. galunisertib)

Response: We appreciated that this reviewer found the last figure is interesting. The EV-TβRII levels were shown to correlate with chemoimmunotherapy resistance in patients (Figure 4a-e). Particularly in PTX non-responders, the percentage of IFN-γ^+ $CD8^+$ T cells was inversely correlated with circulating EV-TβRII (Figure 4d). Moreover, 4T1-bearing balbc mice model showed that loss of USP8 inhibited $CD8^+$ T exhaustion and enhanced anti-tumor immunity (Fig. 5h-n). These analysis combined together suggest that antagonising USP8 might have the potential to simultaneously prevent tumor progression and exhaustion of $CD8^+$ T cells when combined with anti-PD-L1, thereby reactivating anti-tumor immunity. In response to this reviewer, we provided the rationale of combining PDL1 and focusing on T cells (highlighted in Page 20).

In response to reviewer, we performed T Cell-mediated tumor killing assay with combination with the exosome biogenesis/release inhibitor GW4869. As shown, α-PD-L1 together with USP8 inhibitor strongly amplified the tumor-killing capacity of T cells, and this effect was barely affected by GW4869. In contrast, GW4869 can further enhance the tumor-killing efficiency by α-PD-L1 and galunisertib (*Figure for reviewer only 4*). Combined these observations together, USP8 inhibitor not only repressed

TGF-β/SMAD signaling as typical TGF-β receptor kinase inhibitor, but also play role to prevent CD8^{+} T cell exhaustion by alleviating EV-TβRII.

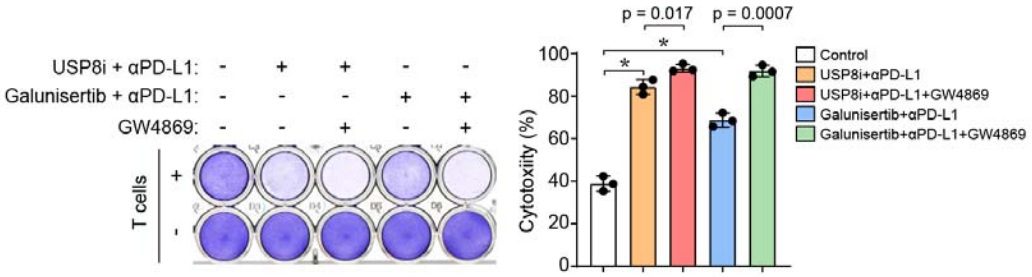

Figure for reviewer only 4: **T cell-mediated cancer cell killing assay. MDA-MB-231 cells co-cultured with activated T cells for 48 hrs with or without USP8 inhibitor (5 μM), anti-PD-L1 (100 ng/ml), galunisertib (10 μM), GW4869 (5 μM) were subjected to crystal violet staining. MDA-MB-231-to-T cell ratio, 1:5. Data represent mean ± SD. n = 3.**

Referee #2:

The manuscript by Xie et al., investigates the role that ubiquitin-specific peptidase 8 (USP8) plays in stabilizing the TGF-beta type II receptor (TβRII), which enhances TGFβ signaling within cancer cells and promotes an epithelial-to-mesenchymal transition (EMT) and breast cancer cell migration/invasion. In addition, USP8 increases the amount of TβRII that is packages into extracellular vesicles (EVs), which can suppress T cell activation and anti-tumor immunity. Interfering with USP8 function impairs TGFβ signaling and diminishes the number of TβRII+ EVs, which leads to increased T cell mediated clearance of breast cancer cells. The authors have built a compelling story that is supported by a significant amount of biochemical, cell biology and in vivo tumor growth and metastasis data. When possible, the authors have examined correlations between USP8 levels and the degree of TGFβ signaling using IHC analyses of human clinical samples and publicly available gene expression data from breast cancer patients. The conclusions drawn are well-supported by the data and will be of interest to the broader cancer community.

Response: Many thanks for such nice summary of our work and the very positive comments. As for the rest major points, please find our detailed responses below.

Specific Points:

The authors suggest that stable knockdown of USP8 diminishes the stem cell characteristics of breast cancer cells - have specific stem cell markers been assessed in the various breast cancer models that do or do not express USP8?

Response: In response to reviewer, we used fluorescence-activated cell sorting (FACS) to assess specific stem cell markers CD44 and CD24 in MDA-MB-231 breast cancer cells. Stable knockdown of USP8 induced *en masse* reduction of the $CD44^{high}/CD24^{low}$ population (see revised Supplementary Fig. 1f, g), suggesting that USP8 can induce the stem cell characteristics of breast cancer cells. Related descriptions were highlighted in Page 6. Many thanks for this suggestion.

The authors suggest that USP8 levels are correlated with mesenchymal markers and that USP8 is required for the TGFβ induced expression of EMT markers in certain cell models. Is loss of USP8 sufficient to induce a mesenchymal-to-epithelial transition in basal breast cancer cells?

Response: In response to reviewer, we analyzed the influence of USP8 loss on well-established MET-related makers. Upon konckdown of USP8, typical MET changes, including downregulation of N-cadherin and vimentin and upregulation of E-cadherin, were not significantly observed in MDA-MB-231 basal breast cancer cells (*Figure for reviewer only 5*). These results indicated that loss of USP8 is not sufficient to induce a mesenchymal-to-epithelial transition in basal breast cancer cells.

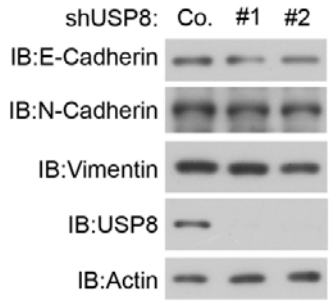

Figure for reviewer only 5: **Immunoblot analysis of cell lysate of control and USP8 stably depleted- MDA-MB-231 cells.**

It appears that endogenous USP8 expression in MDA-MB-231 cell is quite low (Fig. S1b) relative to other basal breast cancer models. It also seems that some of the knockdown studies performed in the MDA-MB-231 model were done in cells engineered to overexpress USP8 (Figure 2i). Is the endogenous level of USP8 in MDA-MB-231 cells sufficient to have an impact on endogenous TGFβ signaling? Is the data shown Fig. 1h a knockdown of endogenous USP8 in MDA-MB-231 cells? It would help for the authors to clarify this point. Also, have USP8 knockdowns been performed in other basal breast cancer models that express higher levels of USP8 (MDA-MB-436) been performed?

Response: The result shown in Supplementary Figure 1b measured the DUB activity, not the total expression of USP8.

We apologized for the typo mistake in Figure 2i, the "Flag" should be "USP8" .

As shown in Figure 2i left panel, depletion of endogenous USP8 is sufficient to antagonize the TGF-β-induced SMAD activation. We also showed in zebrafish embryo xenograft invasion model and mouse xenograft metastasis model that USP8 knockdown inhibited metastasis (Figure 1f-i). Figure 1h indeed showed results from knockdown of endogenous USP8, we made this clear in the text (Page 7) and legend (highlighted in Page 42). 4T1 breast cancer cells are derived from a typical basal-like breast cancer and exhibit a triple-negative phenotype. Knockdown of USP8 was also performed in 4T1 model (Figure 5h-n).

The authors suggest that overexpression of USP8 is correlated with the machinery that produces EVs. They also show that high USP8 levels are correlated with an increase in the number of TβRII+ EVs. Given that loss of USP8 results in a 25% decrease in overall EV secretion but a dramatic reduction in the formation of TβRII+ EVs, do the authors believe that USP8, through removal of ubiquitin from TβRII, results in a greater receptor pool that can be packaged into EVs, or that USP8 directly binds to TβRII and actively chaperones TβRII into EVs?

Response: USP8 directly deubiquitinates TβRII (Figure 3c-f), increases half-life and accumulation of TβRII (Figure 3g-h), sustains TβRII levels on the plasma membrane (Figure 3i-j), thereby leading to enhanced EV-TβRII secretion (Figure 5f-g). These results together indicated that USP8 maintains a greater receptor pool that can be packaged into EVs.

In our analysis, USP8 was not identified in mass spec of EVs from breast cancer cells. In response to reviewer, we confirmed this result by immunoblot analysis (*Figure for reviewer only 6*).

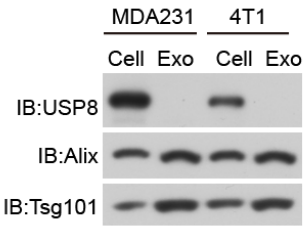

***Figure for reviewer only 6*: Immunoblot analysis of cell lysate and exosome from MDA-MB-231 cells and 4T1 cells.**

The inhibitor caused a 25% reduction in total vesiculation, which was comparable to what was seen in the USP8 knockdown cells. Does the inhibitor show any effect on total vesiculation in cells with a USP8 knockdown - these experiments would reveal any potential off target effects of the UPS8i.

Response: This is a perfect suggestion. In response to reviewer, we compared the total concentration of EVs from control and USP8-depleted 4T1 or MDA-MB-231 cells that were treated with or without USP8 inhibitor. Whereas the USP8 inhibitor significantly reduced vesiculation in control cells, it barely have effect in USP8-depleted cells (*Figure for reviewer only 7*). These results indicated that USP8 should be the primary target of this inhibitor at the dosage used.

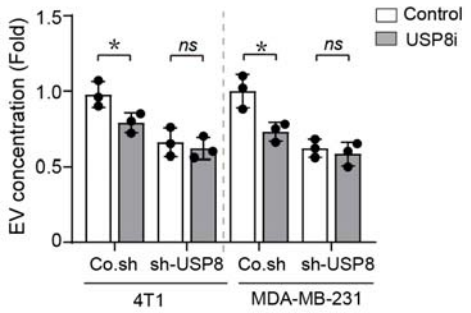

***Figure for reviewer only 7*: The total concentration of EVs by nanosight analysis from 4T1 and MDA-MB-231 cells infected with lentivirus encoding control shRNA (Co.sh) or USP8 shRNA treated with or without USP8 inhibitor (5 μM) for 48 h.**

Can the authors revisit the USP8 staining in the breast tumor TMA to see if high USP8 levels correlate with fewer infiltrating CD8+/GZMB+ T cells (immune cold) relative to tumors that express lower levels of USP8 (immune hot)? Or can the gene expression data be analyzed via a CYBERSORT approach to estimate immune cell populations (CD8+ T cells) in USP8 high or low breast tumors?

Response: In response to reviewer, we analyzed the gene expression data via the CYBERSORT approach. In CIBERSORT analysis, USP8-high breast cancer tumors are associated with low infiltration of $CD8^+$ T cells (see revised Supplementary Figure 8g). The related descriptions were highlighted in Page 21-22. Many thanks for this suggestion.

The authors suggest two possible roles for USP8 in enhancing the ability of breast cancer cells to become more metastatic. The first is through elevated levels of the TβRII, which enhances TGFβ signaling and increased stemness, migration/invasion and metastasis (tumor intrinsic). This clearly occurs in xenograft models where T cell anti-tumor immunity is not a factor (MDA-MB-231). The second is through the potential action of TβRII+ EVs on the suppression of T cell mediated anti-tumor immunity. Do the authors have a sense of what is the predominate mechanism at play in the syngeneic model (4T1). If 4T1 cells are injected into an immunocompromised background, does the USP8i still impair tumor growth (as seen in Balb/c mice - Fig. 7a, b)? If TβRII+ EVs are harvested and injected into Balb/c mice bearing 4T1 breast tumors - does this increase tumor

growth and metastasis relative to TβRII- EVs?

Response: In response to reviewer, we performed new mice experiment. Antibody-based depletion of $CD8^+$ cells significantly promoted 4T1 tumor growth and metastasis; In this background, USP8 inhibitor could still reduce tumor growth, showing that both mechanisms are important (Supplementary Figure 9a-d). The related descriptions were highlighted in Page 23.

To investigate whether exogenously introduced EV-TβRII could promote tumor growth and metastasis, we transplanted 4T1 cells in mice followed by tail vein injections of the in vitro collected TEVs from either their WT or TβRII null counterparts (see revised Supplementary Figure 7e). Injection of EVs from the WT ($TβRII^+$ EVs), but barely the TβRII null ($TβRII^-$ EVs), cells more strongly promoted tumor growth (Supplementary Figure 7f). The circulating EVs from plasma of mice were also elevated after $TβRII^+$ EVs treatment (Supplementary Figure 7g). In line with the tumor growth, the levels of cancer metastases were strongly elevated by $TβRII^+$ EVs, but barely the $TβRII^-$ EVs (Supplementary Figure 7h). Together, these results revealed that $TβRII^+$ EVs increase tumor growth and metastasis *in vivo*. The related descriptions were highlighted in Page 20. Many thanks for this suggestion.

Referee #3:

Xie et al. uncover a deubiquitinating enzyme (DUB) responsible for stabilization of TBRII at the cell surface, thereby influencing EMT and cancer stemness properties, as well as progression of breast cancer to metastatic disease in vivo. High levels of this DUB, USP8, are found in patient tumor samples compared to normal tissue and correlate with poor metastasis free survival. Additionally, they find that reducing the levels of TBRII at the surface influences the quantity of TBRII in circulating extracellular vesicles (EVs), which correlates with reduced primary and metastatic tumor burden and alters expression of several proteins in T cells. A USP8 inhibitor, both alone and synergistically with paclitaxel and anti-PD-L1 therapy, reduces tumor burden and increases active CD8+ T cells. Overall, this is a very thorough and interesting paper with a few conclusions that could be strengthened.

Response: Many thanks for support and favorable comments. Please see our point to point answers below.

Major concerns:

Is it the increased EV-TBRII or increased TBRII in primary/circulating/metastatic tumor cells that is responsible for changes in disease progression? Much of the evidence presented is correlative and not causative for EV involvement. The manuscript generally does not directly state that the EVs are responsible for the outcomes observed, however the title of the manuscript does make this conclusion. To provide direct evidence, could the authors co-culture TBRII+EVs with cancer cells and see a change in migration/invasion for example or with T cells to look for activation and proliferation changes? These experiments of course cut out the complex in vivo environment. In the materials and methods section the authors mention an "EVs education experiment..." on page 25. Have the authors attempted to inject altered EVs into mice to look for changes in a syngeneic manner? This would be a powerful experiment to include to address the direct role of EVs and support the conclusions drawn in the title.

Response: In response to reviewer, we co-cultured TβRII$^+$ EVs with MDA-MB-231 cancer cells and performed transwell assays with or without matrigel to evaluate the effect of TβRII$^+$ EVs on the migration and invasion capacities of cancer cells. Transwell assay indicated that TβRII$^+$ EVs significantly enhanced the migration and invasion of MDA-MB-231 cells either with or without TGF-β treatment, whereas no big differences were found when cells were co-cultured with co.EVs and TβRII$^-$ EVs (Supplementary Figure 7a-b).

We also co-cultured T cells with TβRII$^+$ EVs and measured the T cell proliferation and tumor-killing capabilities. The TβRII$^+$ EVs-induced suppression of T cell proliferation and tumor-killing ability were shown (Supplementary Figure 7c-d).

Please see our answer to reviewer 2, the last question. The new mice experiment demonstrated that TβRII$^+$ EVs could promote tumor growth and metastasis (Supplementary Figure 7e-h). The related descriptions were highlighted in Page 20. Many thanks for this suggestion.

USP8, of course, has target proteins other than TBRII that likely are contributing to the observed outcomes. In the literature, it is demonstrated that EGFR and HIF1a are both validated targets. Can the author's perform experiments that either re-express or knockdown TBRII in USP8

modulated cells to determine the relative impact of TBRII on the overall effect of USP8? In vivo tumorigenic and metastatic studies would be most convincing.

Response: In response to reviewer, we performed new batch of mice experiment (Supplementary Figure 5e-g). The control and USP8-depleted 4T1 cells were transplanted in mice followed by tail vein injection of the *in vitro* collected TEVs from either their WT or TβRII null counterparts. We observed that knockdown of USP8 significantly reduced tumor growth and the lung metastasis; As shown, education with TβRII-containing EVs effectively rescued (although not completely) the reduced tumor growth and metastasis in mice inoculated with USP8-depleted 4T1 cells. These results indicate that TβRII$^+$ EVs have significant impact on the overall effect of USP8. Related descriptions were made and highlighted in Page 16. Many thanks for this suggestion.

Minor concerns:

• In Fig. 1d, what diameter cut off is used to count mammospheres?

Response: The number of the mammospheres with diameter > 60 μm was quantified in Fig. 1d. We have added this detail information into the legend of Fig. 1d (highlighted in Page 41).

• Were any metastatic lesions observed in the in vivo experiment depicted in Fig. 1e?

Response: There were no metastatic lesions from control and USP8-depleted MCF10A-RAS cells within the time scale (5 weeks).

• What method is used in Fig. 2h? I can't find where it is clearly described whether this is qPCR or luciferase data.

Response: We apologized for this irrelevant description. Fig. 2h showed results from qPCR array and we made this clear in the legend (highlighted in Page 43).

• What is Flag tagged in Fig. 2i left panel?

Response: Many thanks for pointing out this typo for us, and it should be USP8. We corrected this mistake.

• Add another mesenchymal marker such as vimentin to the immunofluorescence panel in 2j.

Response: The vimentin staining was included as Supplementary Fig. 2c.

• On page 10 an experiment was described with flag-tagged TBRII and HA-ubiquitin but I cannot find this experiment in the figures.

Response: That was a description on how we harvested poly-ubiquitinated TβRII as substrate used for Figure 3c.

• Was ubiquitination of TBRI by USP8 also investigated and ruled out?

Response: This has been tested and USP8 has no effect on the ubiquitination of TβRI.

• Please add legends to 4f panel 2 and 4g.

Response: The legends were added (highlighted in Page 45-46).

• 'Gene signatures representing vesicle formation and cancer metastasis' were mentioned in the text describing Figure 5a, however metastasis is not shown. Could this be added/ corrected?
Response: We apologized for this irrelevant description and it has been removed.

• When was inhibitor treatment initiated in Fig. 6k?
Response: USP8 inhibitor treatment was initiated on day 1 after tumor inoculation in Fig. 6k. We made this clear in the legend (highlighted in Page 48) .

• On page 16, TBRII mRNA levels are referenced but the figure is not shown.
Response: This was included as Supplementary Fig. 6a.

• Inconsistent spelling (e.g. tumour vs tumor) and other minor typos throughout (e.g. 'bresat' in Figure 6i).
Response: We corrected those mistakes, many thanks for pointing out these typos for us.

Reference

[1] L. Zhang, F. Zhou, Y. Drabsch, R. Gao, B.E. Snaar-Jagalska, C. Mickanin, H. Huang, K.A. Sheppard, J.A. Porter, C.X. Lu, P. ten Dijke, USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor, Nat Cell Biol, 14 (2012) 717-726.

[2] F. Xie, K. Jin, L. Shao, Y. Fan, Y. Tu, Y. Li, B. Yang, H. van Dam, P. Ten Dijke, H. Weng, S. Dooley, S. Wang, J. Jia, J. Jin, F. Zhou, L. Zhang, FAF1 phosphorylation by AKT accumulates TGF-beta type II receptor and drives breast cancer metastasis, Nat Commun, 8 (2017) 15021.

[3] Z. Zhang, Y. Fan, F. Xie, H. Zhou, K. Jin, L. Shao, W. Shi, P. Fang, B. Yang, H. van Dam, P. Ten Dijke, X. Zheng, X. Yan, J. Jia, M. Zheng, J. Jin, C. Ding, S. Ye, F. Zhou, L. Zhang, Breast cancer metastasis suppressor OTUD1 deubiquitinates SMAD7, Nat Commun, 8 (2017) 2116.

Dear Prof. Zhou,

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referees. As you can see below, the referees appreciate the introduced changes and support publication here.

I am therefore very pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal accept letter there are just a few editorial points to resolved:

- Please upload high resolution individual figure single

- The supplementary information should be uploaded as an appendix with a ToC. You can have 5 expanded view figures - please see guide to authors. If you choose to have expanded view figures, please upload them as individual files.

- The email for Huib Ovaa bounced. Please correct

- Make sure that the deposited RNAseq data is open and available.

- The funding also needs to be added to the online submission system.

- Please adjust the reference format to EMBO Journal style

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

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When you upload your revised version, please also prepare a point-by-point response to the editorial points.

That should be all - let me know

Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #1:

This is a revised version of the previously submitted manuscript. The manuscript has clearly improved including new models (including a more clinically related model) and an extended series of observations. As previously stated, the information provided is relevant but had some hurdles to overcome. In this current version this has significantly improved with genetic validations and some experiments that strengthen the authors claims. Se below a few points.

In particular, the experiments showing the effect of the treatment in previously established (and not only de novo) metastatic lesions is relevant. In addition, the experiments clarifying the cancer cell autonomous effects versus the EV-mediated are sound. In line, there are several technicalities for which reasonable answers have been provided, ranging from the suitability of the IHC antibodies used to the relationship between the EV produced compared to basal TGFbeta activity. Finally, the figures modifications, including the new plots for figure 1i.

Finally, the rational for the last figure as well as the overall tone of the manuscript makes it more streamlined and clearer. Still some typos are found within the text (i.e. foundings should be findings, page 20 first line 2nd paragraph). Please double check.

Overall the manuscript has improved through the revision process.

Referee #2:

The revised manuscript by Xie et al. entitled "Targeting USP8 inhibits Cancer Progression and Prevents Extracellular-Vesicle TβRII-Induced Exhaustion of CD8+ T cells" (EMBOJ-2021-108791R) has been strengthened by the additional experiments that have been performed in response to issues raised during the first review. Indeed, the authors have been highly responsive in addressing the reviewer's comments. I support publication of the revised manuscript.

Referee #3:

The authors have addressed my concerns from the original review adequately by completing extensive co-culture experimentation and in vivo administration of TBRII modulated EVs.

Editorial points:

- Please upload high resolution individual figure single

Response: Yes, we have uploaded high resolution individual figure single.

- The supplementary information should be uploaded as an appendix with a ToC. You can have 5 expanded view figures - please see guide to authors. If you choose to have expanded view figures, please upload them as individual files.

Response: We have uploaded the supplementary information as an appendix with a ToC. We also uploaded expanded view figures as individual files.

- The email for Huib Ovaa bounced. Please correct

Response: Dr. Huib Ovaa's passed away because of prostate cancer, and his email may be deactivated.

- Make sure that the deposited RNAseq data is open and available.

Response: Yes, the RNAseq data is open and available (https://ngdc.cncb.ac.cn/gsa-human/browse/HRA002342).

- The funding also needs to be added to the online submission system.

Response: We have added the fundings in the online submission system.

- Please adjust the reference format to EMBO Journal style

Response: Yes, we have adjusted the reference format to EMBO Journal style.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

Response: Yes, we have provided a PDF files containing the original, uncropped and unprocessed scans of key gels labeled with the appropriate figure/panel number and molecular weight markers.

- Can you take a look at the size of synopsis image and see if correct size. It should be 550 wide by [200-400] high (pixels)

Response: We have edited the image to fit the size.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.

Please leave the comments and your reply marked in the text so that I easily can see your response.

Response: Thanks for checking and we have responded to the issues in the Data Edited Manuscript file.

2nd Revision - Editorial Decision **3rd Jun 2022**

Dear Fangfang,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and I am pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist for Life Science Articles (updated January 2022)

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 manuscript.

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 replicates.
- 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 Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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	All antibodies have been described as required in Materials and Methods:Flow cytometry analysis; Immunoprecipitation and immunoblot analysis; Animal studies and mice metastasis models; Immunohistochemical staining and evaluation.

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	Supplementary Table S3 Primers list

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods:Cell culture
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods:Cell culture
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods:Cell culture

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	Materials and Methods: Animal studies and mice metastasis models
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Yes	Materials and Methods: Animal studies and mice metastasis models
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods: Animal studies and mice metastasis models

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: Patients and specimen collection

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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)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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	Materials and Methods: Statistical analyses
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	Materials and Methods: Statistical analyses
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods: Statistical analyses
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.	Not Applicable	
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	Materials and Methods: Statistical analyses

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 legnds and Supplementary Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legnds and Supplementary 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	Materials and Methods: Patients and specimen collection
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.	Yes	Materials and Methods: Patients and specimen collection
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: Animal studies and mice metastasis models
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	

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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.	Not Applicable	
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	Materials and Methods: Data availability
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?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods: Patients and specimen collection