nature portfolio

Peer Review File

Loss of NUMB drives aggressive bladder cancer via a RHOA/ROCK/YAP signaling axis

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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)
In this manuscript, Tucci and colleagues address the contribution of the putative tumor suppressor factor NUMB to bladder cancer (BCa) aggressiveness and progression. The authors exploit a combination of (i) large cohorts of human BCa samples, (ii) Numb-KD human BCa cell lines, (iii) Numb-KO genetically engineered mouse models (CK5-Cre Numblox/stop/lox) and chemically-induced BCa mouse models, and (iv) human cell- and mouse model-derived organoids combined with pharmacological inhibition to investigate the prognostic, clinical, and functional relevance of NUMB in BCa. They mainly show that:
- Low NUMB expression and an associated NUMBless molecular signature correlate with non-muscle invasive (NMIBC) recurrence and progression towards muscle invasive disease (MIBC). Thus, the NUMB-deficient status has prognostic value in both NMIBC and MIBC patients
- In vivo, Numb KO leads to the occurrence of pre-neoplastic and neoplastic lesions (both non-invasive and invasive) as well as to the acceleration of the tumor phenotype in BBN-induced mice (a well-established carcinogen-induced model of BCa). - Mechanistically, Numb loss results in the upregulation of RhoA/ROCK signalling to the actin cytoskeleton, which leads to the consequent hyperactivation of YAP transcriptional activity.
- RhoA/ROCK/YAP signalling is responsible for the aggressive migratory/invasive phenotype of Numb-deficient BCa cells, which can be targeted using inhibitors specific for the different effectors of the pathway.
This is a well written and interesting manuscript which integrates a combination of experimental models and molecular analyses to investigate the role of NUMB in BCa. The role of NUMB in tumorigenesis has been relatively understudied and has not been addressed in the context of BCa. The study therefore brings significant novel insights into mechanisms driving BCa progression and uncover therapeutic vulnerabilities with potential clinical relevance. In my opinion, the study is worthy of publication in Nature Communications provided that the authors address the following points:
1. The contribution of YAP in bladder tumorigenesis has already been suggested in previous studies and it would be good to acknowledge and discuss them (e.g. PMID: 30158528, PMID 35665684); as an example, molecular subgroups stratified according to YAP1 activation are associated with different prognoses and treatment response (BCG) in NMIBC (PMID 35665684). In this publication, they used the same UROMOL cohort as used in Figure 1 of the present study. Is the NUMBless signature a surrogate for YAP activity and therefore would overlap with the YAP associated subtypes highlighted in this paper? Does the NUMB status associate with treatment response and may therefore represent a predictive factor?

2. What is the pattern of expression of Numb in other epithelial tissues? Does Numb loss have any effects on the phenotype of other tissues? As pointed by the authors in the discussion, NUMB is an evolutionarily conserved cell fate determinant with potential tumor suppressor function in several cancer types. Given that Numb loss is driven by the KRT5 promoter which is not bladder specific, it may affect all the other epithelial tissues in the KO mouse model and potentially the survival analyses.

Cre-drivers can have distinct efficiency of recombination depending on the promoter and cell types; what is the % of cells which efficient recombination i.e. with Numb loss of expression in the KO models at the start of the treatment?

3. For the in vivo experiments, what is the rationale for using only male mice ?

4. In breast cancer, NUMB loss of function has been shown to induce increased activity of the oncogene NOTCH. NOTCH has rather be proposed to represent a tumor suppressor gene in BCa even though some studies also suggest an oncogenic role (PMID: 25574842, PMID: 29643502). What is the relationship of NUMB and NOTCH in the context of this study?

Expression of NOTCH following NUMB loss in vitro and in vivo?

5. What are the putative causes of NUMB loss of expression during BCa progression and in a subset of patients? Are there NUMB genomic alterations? This could be easily checked by looking at published datasets. Putative epigenetic mechanisms? The authors also show that Numb is spontaneously lost upon tumor progression (Fig.2, wild type mice) which may imply that this is a consequence of tumor progression rather than a cause (or both, difficult to discriminate in patient cohorts).

Minor comments:
- Page 8: "In mouse and human" while mouse experiments have not been introduced in the text yet
- Double check nomenclature to refer genes vs. proteins in mouse vs. human (small vs. capital, italic vs normal,)
- The "Real-life" in not necessary when referring to BCa patients

Reviewer #2

(Remarks to the Author)
This a very interested and well crafted manuscript on a subject that is both highly significant and understudied. The manuscript by Tucci et al investigates the role of Numb in the transition from non-muscle invasive (NMIBC) to muscle invasive bladder cancer (MIBC). First they show that numb expression is associated with disease outcome in patients, and link this to YAP signaling. They then perform functional studies in mouse models and organoids to investigate these activities. They propose - and present data to support - that this provides a therapeutic opportunity for treating this group of patients that have few treatment options. Overall, the work is of high quality and the manuscript well-written and well presented. This review particularly appreciates the careful analyses of the organoid models. I have only a few comments to further improve this fine study.

1. The rationale for studying Numb in the first place needs to be introduced -- ideally in the introduction and I think also in the first paragraph of the results.

2. The patient analyses regarding NUMB expression and YAP activity is very important and this reviewer thinks could be highlighted more effectively in the Main text. I also think this is a bit confusing to divide into 3 separate sections of the results. I would suggest to move some of the data from the Supplement to the main body and/or split to 2 figures. In particular, it would be beneficial to see outcome data for the YAP - currently the data in the main body only shows the relation of Numb and YAP but not the relevance for patient outcome.

3. Related to the comment above - the gene signature data should be part of the main body as this is most directly translatable.

4. What is the relationship of the current gene signature to previous gene signatures for bladder cancer outcome - this information could be included as supplementary. Here I am thinking to show if there is overlap of the genes in the signatures and/or comparison of their strength -- so, are they complementary and add value to each other OR very similar to each other. This reviewer realizes that most readers of Nature Comm will be interested in the mechanistic figures that follow, but the relationship to bladder cancer outcome is very important and should be better highlighted.

5. Panel 1j - is not very compelling and could be improved.

6. For the mouse model - if this reviewer understands correctly (from the methods), the model has a flexed allele of Numb crossed with a CK5-Cre allele - if so, this will delete numb in all CK5 expressing cells (which are in many tissues). How do the authors know that they effects they are seeing are cell-autonomous to the bladder. If they do not know, they should add a caveat so as not to be misleading. This is especially important for the data in Fig. 2 showing that Numb alone promote a pre-cancerous and even cancerous phenotype in bladder. (very few genes do so).

7. It would be beneficial to name the pathologist making the evaluation of the phenotype (in the methods).

8. I do not see methods for how they made the organoids - including the age, sex, how they are selecting the cells (sorting?) etc. This is a serious omission that needs to be corrected in the methods.

9. Along these lines, I also do not see whether they are looking at both males and females and whether there are differences. This is very important and should be highlighted throughout.

10. Some of the conclusions are stated too strongly and should be toned down. This would not detract at all from the paper. For example (but there are others) -- this statement -- "Thus, Numb status could guide clinical decision-making between conservative vs. more aggressive therapies" is much too strongly stated.

11. Why did they limit analysis of the drug to BBN organoids only? I dont think I missed the data for the non-BBN organoids in the supplement.

12. I think the drug has to be better characterized for its specificity. I see knock-down data in the supplement - but I think this could be more explicitly shown.

13. I find this a bit confusing to conclude the paper with the data in Fig. 7 - which is disconnected to Fig. 1. I wonder if the authors could find a better way to coordinate these findings for improved clarity.

14. Again, while this Reviewer understands that most readers will be most interested in the mechanistic relationships, the value to patients with NMIBC is very important not really highlighted in the discussion. I think this is a missed opportunity and a few sentences would help alot.

Reviewer #3

(Remarks to the Author)
In this manuscript, authors found loss of Numb expression correlates with worse overall survival in post-cystectomy muscle-invasive bladder cancer (MIBC) patients and increased risk of MIBC progression in non-muscle-invasive bladder cancer (NMIBC) patients. Using Numb knockout transgenic mouse model and BBN carcinogen treatment model, authors further demonstrated the tumor suppressor role of Numb in bladder cancer. Using 3D-Matrigel organoid culture model, authors found that Numb loss heightens the proliferative and invasive potential of both mouse and human bladder cancer (BCa) cells. Integrative transcriptomic and functional analyses revealed that downregulation of the canonical Hippo pathway, resulting in enhanced YAP transcriptional activity, underlies the biological aggressiveness of Numb-deficient BCa. These molecular events are dependent on the activation of RhoA/ROCK signaling subsequent to Numb loss. Thus, a dysfunctional Numb–RhoA/ROCK–Hippo/YAP regulatory network is at play in aggressive Numb-deficient BCa and represents a therapeutic vulnerability.

The manuscript was well-written, and findings are interesting.
Major points:
1. The immunoblot of Numb sometimes showed two bands, sometimes showed one band. Can authors explain this discrepancy?

2. It is unclear the time frame of Bca tumor onset in Numb-knockout mice.

3. Figure 3a-b, why the WT 3D organoid structures were so different? Figure 3b showed multi-acini structure in Numb-KO, what is frequency of this phenotype?

4. The relationship between EMT and VP mentioned but was not further discussed.

5. The study is not clear about the specific regulatory mechanisms of NUMB, RhoA/ROCK/YAP signaling pathways. Figure5, regarding the activation of the Hippo pathway by Numb through the inhibition of the RhoA/ROCK signaling pathway, which in turn affects the transcriptional activity of YAP, a process that correlates with the aggressiveness and progression of BCa. However, the details of how RhoA/ROCK signaling is specifically regulated and the role of Numb deficiency with other potential signaling pathways are not yet clear. Are further experiments planned in follow-up, such as using mass spectrometry, to reveal the direct interactions between Numb and these signaling molecules?

6. Numb is a crucial determinant of cell fate, involved in regulating processes such as cell differentiation and proliferation. It may potentially influence the function of the endoplasmic reticulum and protein folding, which could in turn affect the expression and activity of GRP94, a molecular chaperone of the endoplasmic reticulum. It is not clear why authors used GRP94 as a loading control in Immunoblot analysis.

7. Lack of evidence of dysfunction/activation of YAP/TAZ in bladder cancer patients

Minor points:

1. Figure 1j, authors described the correlation between low expression of Numb protein and increased nuclear accumulation of YAP. However, figure 1j showed YAP positive versus YAP negative.

2. Figure 3E, the BBN-KO 3D organoid image quality needs to be improved.

3. Would it be possible to arrange Figure 2 & 5 in a landscape orientation?

4. Extended Data Fig. 5a, Supplementary Table 9, see also Supplementary Table 10 for a complete list of differentially regulated genes), which was also sensitive to VP treatment (Extended Data Fig. 5a, Supplementary Table 11). This part of the RNA-seq results is seen in the Supplementary Fig. Although GSEA analysis provides gene expression patterns, it is best to combine it with specific biological function experiments to enhance the biological significance of the results. (In this study, the authors did not design specific functional experiments to directly verify the role of YAP target genes in the EMT process).

5. The spacing between the target bands in the immunoblot analysis of Figure 4B appears uneven.

6. Some of the references are incorrectly formatted, e.g:13、14、32、34 et al. Kindly verify and make the necessary adjustments with careful attention.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have significantly improved the manuscript and thoroughly addressed my comments, as well as the critics of the two other reviewers. I would like to congratulate them for the amount of work and the high quality of their manuscript, which is elegant both in terms of content and data/figures quality. I believe that the manuscript is worthy of publication in Nature Communications provided that the below minor comments are further addressed/discussed:

- "Therefore, the NUMBLESS signature could be used to identify patients with tumors displaying aberrant YAP/EMT activation (line 209)". The therapeutic and translational relevance of the NUMBless signature is also discussed in the discussion part.

If I understood correctly, the authors show that the YAP activation signature has more prognostic/predictive significance than the NUMBless signature (fig. 2f and 1h). What would be the advantage to use a NUMBless signature versus the YAP activation signature then? Is it adding some information or is it "easier" to assess? What about the combination of them? It is difficult to grasp the translational potential specifically related to NUMB in view of the important prognostic/predictive significance of the YAP signaling per se and the fact that YAP is targetable while NUMB is likely not (which does not diminish the mechanistic significance of the study). Thus, I would be careful with such type of statement or come up with a better rationale underlying the translational potential associated with NUMB findings which add value to the known significance of YAP in BCa.

- Related to non-bladder specific effects of Numb KD in CK5-positive cells:

I appreciate the careful examination of the authors in the various organs. Yet it is somehow difficult to understand that targeted Numb silencing in the basal layer of the mouse urothelium is alone sufficient to trigger spontaneous bladder tumorigenesis, while it has 0 effects on other CK5+ tissues (knowing that Numb has been shown to have a role in some of these tissues). The authors discuss results in the breast which can be justified by the differences between males and females. In contrast, it is surprising to observe no effects (after 24 weeks) in male organs such as the prostate (i) which has a full CK5+ basal layer and (ii) in which NUMB has been demonstrated to be potentially important for tumorigenesis. Any explanation?

- Please, double-check the few typos and conjugation mistakes throughout the text (e.g.
Line 433 "Supporting this hypothesis, treatment with Y-27632 reversed the aberrant morphology of NUMB-KO MBOs and markedly decreased YAP transcriptional activity (Supplementary Fig. 9i), while have no significant effects on WT MBOs...)

Reviewer #2

(Remarks to the Author)
In the previous version, the study by Tucci et al had provided important new insights into the role of NUMB in bladder cancer. The revised manuscript, in this reviewer's opinion, has been greatly improved by addressing the comments of all three reviewers. This is an outstanding revision and outstanding manuscript. I have no further suggestions.

Reviewer #3

(Remarks to the Author)
I and co-reviewer agreed that authors have successfully addressed the reviewers' comments.

We recommend the manuscript to be accepted for publication.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature

Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

POINT BY POINT REPLY TO THE REVIEWERS' COMMENTS (reproduced verbatim)

Reviewer #1

In this manuscript, Tucci and colleagues address the contribution of the putative tumor suppressor factor NUMB to bladder cancer (BCa) aggressiveness and progression. The authors exploit a combination of (i) large cohorts of human BCa samples, (ii) Numb-KD human BCa cell lines, (iii) Numb-KO genetically engineered mouse models (CK5-Cre Numblox/stop/lox) and chemically-induced BCa mouse models, and (iv) human cell- and mouse model-derived organoids combined with pharmacological inhibition to investigate the prognostic, clinical, and functional relevance of NUMB in BCa. They mainly show that:

- Low NUMB expression and an associated NUMBless molecular signature correlate with non-muscle invasive (NMIBC) recurrence and progression towards muscle invasive disease (MIBC). Thus, the NUMB-deficient status has prognostic value in both NMIBC and MIBC patients.

- In vivo, Numb KO leads to the occurrence of pre-neoplastic and neoplastic lesions (both noninvasive and invasive) as well as to the acceleration of the tumor phenotype in BBN-induced mice (a well-established carcinogen-induced model of BCa).

- Mechanistically, Numb loss results in the upregulation of RhoA/ROCK signalling to the actin cytoskeleton, which leads to the consequent hyperactivation of YAP transcriptional activity.

- RhoA/ROCK/YAP signalling is responsible for the aggressive migratory/invasive phenotype of Numb-deficient BCa cells, which can be targeted using inhibitors specific for the different effectors of the pathway.

This is a well written and interesting manuscript which integrates a combination of experimental models and molecular analyses to investigate the role of NUMB in BCa. The role of NUMB in tumorigenesis has been relatively understudied and has not been addressed in the context of BCa. The study therefore brings significant novel insights into mechanisms driving BCa progression and uncover therapeutic vulnerabilities with potential clinical relevance. In my opinion, the study is worthy of publication in Nature Communications provided that the authors address the following points.

R. We thank Reviewer #1 for the appreciative words and for highlighting the novelty of our findings establishing the relevance of NUMB as a tumor suppressor in bladder tumorigenesis and disease progression, and their translational potential. We also thank this Reviewer for the suggestions provided, which helped us to craft an improved version of our manuscript.

This Reviewer raised several highly relevant points that we address below *(the points are reproduced verbatim, maintaining the reviewer's original numbering for convenience)*:

1) *The contribution of YAP in bladder tumorigenesis has already been suggested in previous studies and it would be good to acknowledge and discuss them (e.g. PMID: 30158528, PMID 35665684); as an example, molecular subgroups stratified according to YAP1 activation are associated with different prognoses and treatment response (BCG) in NMIBC (PMID 35665684). In this publication, they used the same UROMOL cohort as used in Figure 1 of the present study. Is the NUMBless signature a surrogate for YAP activity and therefore would overlap with the YAP associated subtypes highlighted in this paper? Does the NUMB status associate with treatment response and may therefore represent a predictive factor?*

R. We thank the Reviewer for raising this relevant point. In the revised manuscript, we have acknowledged and discussed previous studies, including those specifically mentioned by the Reviewer, indicating a contribution of YAP to BCa: *PMID: 35665684* (S. W. Baek, et al., *YAP1 activation is associated with the progression and response to immunotherapy of non-muscle invasive bladder cancer*. EBioMedicine, 81:104092, 2022); *PMID 35665684* (M. K. Gill, et al., *A feed forward loop enforces YAP/TAZ signaling during tumorigenesis.* Nat Commun, 9:1,3510, 2018); *PMID:*

23870412 (J. Y. Liu, et al., O*verexpression of YAP 1 contributes to progressive features and poor prognosis of human urothelial carcinoma of the bladder*. BMC Cancer, 13:349, 2013) (see page 10, lanes 194-197 and lanes 199-202 in the Results of the revised manuscript). To address the specific points raised by this Reviewer, we performed a systematic analysis in the UROMOL cohort of 535 NMIBC patients (Lindskrog *et al. An integrated multi-omics analysis identifies prognostic molecular subtypes of non-muscle-invasive bladder cancer*. Nat Commun, 2021;12(1):2301, *PMID: 33863885*) to investigate the relationship between NUMB dysfunction and YAP activation relative to prognosis and response to BCG immunotherapy. In particular, we analyzed the rate of muscle-invasion progression and BCG response in patients stratified by the $NUMB^{LESS}$ signature or a published 22-gene YAP signature extensively used in the literature to assess YAP activation status in various types of human cancers, including BCa (Wang, X. et al., *Comprehensive Molecular Characterization of the Hippo Signaling Pathway in Cancer*. Cell Rep 2018; *PMID: 30380420*). The YAP signature was consistently employed throughout our study to analyze the transcriptomic profiles from our human and mouse BCa models. Overall, the results of these analyses (see new Figure 2, new Supplementary Figure 3, and page 11, lanes 204-210 of the revised manuscript) support the Reviewer's idea that the $NUMB^{LESS}$ signature behaves as a surrogate of an active YAP status, similarly predicting increased rate of progression and positive response to BCG immunotherapy.

Specifically, in the revised manuscript, we provide evidence of the following points:

a) High expression of the YAP signature predicts: *i*) enhanced risk of muscle invasion independently of clinical pathological parameters in a multivariable analysis (new Figure 2f and new Supplementary Figure 3c), and *ii*) positive response to BCG immunotherapy (new Supplementary Figure 3d). Of note, these results are in keeping with a previous study reporting an association between active YAP status and enhanced risk of muscle-invasion progression and positive response to BCG in NMIBC patients (S. W. Baek, et al., *YAP1 activation is associated with the progression and response to immunotherapy of non-muscle invasive bladder cancer*. EBioMedicine, 81:104092, 2022; *PMID 35665684*).
b) Active YAP status, as assessed by the YAP signature, is significantly associated with the $NUMB^{LESS}$ -like status, indicative of NUMB dysfunction (new Figure 2g), establishing the relevance of NUMB loss/YAP activation, originally characterized in our human and mouse models, to human BCa disease.
c) Similarly to the YAP signature, high expression of the $NUMB^{LESS}$ signature is an independent predictor of muscle invasion progression (previously reported in Figure 1h and Extended Figure 2d of the original version of the manuscript, and now reported in new Figures 1h,i). In addition, the $NUMB^{LESS}$ signature significantly associates with a positive response to BCG immunotherapy (new Supplementary Figure 3e).

2) *What is the pattern of expression of Numb in other epithelial tissues? Does Numb loss have any effects on the phenotype of other tissues? As pointed by the authors in the discussion, NUMB is an evolutionarily conserved cell fate determinant with potential tumor suppressor function in several cancer types. Given that Numb loss is driven by the KRT5 promoter which is not bladder specific, it may affect all the other epithelial tissues in the KO mouse model and potentially the survival analyses.*
R. We acknowledge the Reviewer's point, which is similar to point 6 raised by Reviewer #2. While it is possible that the homeostasis of other CK5-positive tissues with physiological NUMB expression may be subverted in the CK5-NUMB-KO model, we believe that it is highly unlikely for the following reasons:
a) Our spontaneous and BBN-induced bladder tumorigenesis studies comparing CK5-Cre/NUMB-KO *vs.* CK5-Cre-WT mice employed exclusively male mice. A systematic histopathological analysis of all CK5+ tissues characterized by physiological NUMB expression in these mice, revealed no evident signs of aberrant morphology, with the exception of the bladder mucosa. This includes the mammary gland which, in female mice, exhibits preneoplastic alterations in CK5-Cre/NUMB-KO mice (D. Tosoni et al., *The Numb/p53 circuitry couples replicative self-renewal and tumor*

suppression in mammary epithelial cells. J Cell Biol 2015: 211, 4: 845-62; *PMID: 26598619*). This latter finding is not unexpected given that the male mammary gland appeared almost completely substituted by adipose tissue. These results indicate that confounding effects on our *in vivo* studies and survival analyses due to other organ lesions are highly unlikely. These data are now shown in Supplementary Fig. 5a,b of the revised manuscript.

b) In the BBN studies, all BBN-treated CK5-NUMB-KO and control CK5-Cre-WT mice showed, as expected, infiltrating bladder tumors at autopsy performed at the human endpoint, with a maximum latency period of ~60 weeks for control CK5-Cre WT mice and a much shorter maximum latency (~40 weeks) in CK5-NUMB-KO mice. In contrast, no differences were observed in the survival rates of control CK5-NUMB-KO *vs.* CK5-Cre-WT mice treated with 0.05% DMSO in regular drinking water (see the dashed blue and red lines representing BBN treatment-naïve CK5-NUMB-KO and CK5-Cre WT mice, respectively, in the new Figure 3d – previously old Figure 2d). This finding argues that NUMB loss *per se*, either in the bladder or in any other CK5+ tissue, does not affect the overall survival of mice, at least in the observation period considered in our experiments.

c) BBN has been long documented as a chemical carcinogen specific to the bladder mucosa, with no tumor lesions described in other organs (Bertram JS and Craig AW. *Specific induction of bladder cancer in mice by butyl-(4-hydroxybutyl)-nitrosamine and the effects of hormonal modifications on the sex difference in response*. Eur J Cancer, 1972; *PMID: 4651993*).

Based on these considerations, we conclude that, despite the possibility that CK5-driven NUMB ablation might perturb the homeostasis of other epithelial tissues, as we previously observed in the female mammary gland, it is highly unlikely that in our specific experimental setting, the survival rate of BBN-treated CK5-NUMB-KO male mice is influenced by alterations depending on the loss of the homeostatic function of NUMB in other CK5-expressing tissues.

Cre-drivers can have distinct efficiency of recombination depending on the promoter and cell types; what is the % of cells which efficient recombination i.e. with Numb loss of expression in the KO models at the start of the treatment?

R. Acknowledged. In the new Supplementary Figure 5d, we show NUMB IHC analysis of the bladder mucosa in a set of fourteen ~8 week-old mice (the age at which BBN treatment was started). A NUMB-KO efficiency ranging from 50 to 100% was observed in 64% of the mice, with 6 of the 14 mice showing 90-100% NUMB-KO efficiency. Only 3 of the 14 mice (~20%) exhibited a NUMB-KO efficiency <30%. Importantly, when hyperplastic lesions were observed, they displayed complete loss of NUMB expression, as shown in the representative IHC image in new Supplementary Figure 5d.

3) *For the in vivo experiments, what is the rationale for using only male mice?*

R. We thank the Reviewer for highlighting the need to clarify this choice. As mentioned above in point #2, male rather than female mice were chosen for the *in vivo* studies to guard against possible confounding effects linked to the appearance of preneoplastic/neoplastic lesions in the mammary gland (D. Tosoni et al., *The Numb/p53 circuitry couples replicative self-renewal and tumor suppression in mammary epithelial cells*. J Cell Biol 2015: 211, 4: 845-62; *PMID: 26598619*). Furthermore, male mice are known to be more susceptible to BBN-induced bladder tumorigenesis, with the appearance of earlier morphological alterations and reduced survival compared to female mice (Bertram JS and Craig AW. Specific induction of bladder cancer in mice by butyl-(4-hydroxybutyl)-nitrosamine and the effects of hormonal modifications on the sex difference in response. Eur J Cancer, 1972; PMID: 4651993). Therefore, we reasoned that male mice would represent a more suitable model for comparing the kinetics of appearance of morphological urothelial alterations and disease evolution in WT and NUMB-KO mice. This information is highlighted in the revised manuscript (see page 11-12, lanes 226-237 and 242-244).

However, this point, which is similar to point 9 of Reviewer #2, raises the question of whether our findings are relevant to both male and female human BCa. We have obtained several lines of evidence indicating that our results are germane to both male and female BCa:

a) The identification of the NUMB loss/YAP/EMT connection and the derivation of the NUMB$_{LESS}$ signature were based on molecular profiling studies performed in human BCa cell lines of both male and female origin. This information is highlighted in the revised manuscript (see page 7, lanes 118-122; page 8, lanes 128-130)

b) Validation studies of the actionability of the RHOA/ROCK/YAP axis to reverse EMT and acquisition of aggressive proliferative and invasive/migratory phenotypes were performed in both male (RT4) and female (RT112) human cell lines, yielding mirroring results (see page 21, lane 474-479; page 24, lanes 546-550; page 27, lanes 623-625).

c) The prognostic value of NUMB loss, assessed by IHC, in MIBC and NMIBC patients is independent of standard clinical variables, including sex (see Figure 1b,c and Supplementary Figures 1a-d of the revised version; see also page 6, lanes 86-95). Likewise, the NUMB^{LESS} signature is an independent predictor of risk of muscle-invasion progression in the UROMOL cohort, where NUMB^{LESS}-like patients (i.e., with a dysfunctional NUMB status) are equally distributed among males and females (see new Supplementary Figure 1e; see also page 8, lanes 131-141).

d) Our data generated in male mice should also be considered for their relevance to naturally occurring human BCa disease in light of evidence that BCa incidence and mortality rates are disproportionately higher in men compared to women by a 4:1 ratio (Zhang Y. et al. *The global landscape of bladder cancer incidence and mortality in 2020 and projections to 2040*. J Glob Health. 2023; 13: 04109; *PMID: 37712386*).

Together, these results suggest that the underlying biology associated with a NUMB dysfunctional status most likely plays an indistinguishable role in the neoplastic transformation of the bladder urothelium in both males and females. However, in the Discussion of the revised manuscript, we raise the potential limitation of the exclusive use of male mice for our *in vivo* studies and discuss the evidence supporting the relevance of our findings to both male and female human BCa (see page 27, lanes 621-635).

4. *In breast cancer, NUMB loss of function has been shown to induce increased activity of the oncogene NOTCH. NOTCH has rather be proposed to represent a tumor suppressor gene in BCa even though some studies also suggest an oncogenic role (PMID: 25574842, PMID: 29643502). What is the relationship of NUMB and NOTCH in the context of this study? Expression of NOTCH following NUMB loss in vitro and in vivo?*

R. We acknowledge this Reviewer's point. Based on our previous studies in the mammary gland showing an inverse relationship between NUMB dysfunction and NOTCH activation, we have investigated in-depth the relevance of this connection to NUMB-deficient bladder tumorigenesis in our human and mouse BCa models, as well as in BCa patients. Together, our results indicate that a dysfunctional NUMB status in the bladder is associated with downregulation, rather than hyperactivation, of NOTCH signaling. This finding supports a tumor suppressor function of NOTCH in the homeostasis of the bladder mucosa. Our results regarding NOTCH are summarized below:

a) We performed a GSEA of the transcriptomic profiles of the NUMB-proficient human cell lines (RT4, RT112 and KK47), silenced or not for NUMB (NUMB-KD *vs.* Ctr-KD), using a signature widely used in the literature to score NOTCH signaling activation in different experimental settings (C. Giachino et al., *A Tumor Suppressor Function for Notch Signaling in Forebrain Tumor Subtypes*. Cancer Cell, 28,6:730–42, 2015; *PMID: 26669487*; N.J. Robinson et al., *SLX4IP Promotes RAP1 SUMOylation by PIAS1 to Coordinate Telomere Maintenance through NF-κB and Notch Signaling*. Science Signaling, 14,689, 2021; PMID: 34187905; M. Minutti et al., *Distinct Ontogenetic Lineages Dictate cDC2 Heterogeneity*. Nature Immunology, 25,3: 448–61, 2024; *PMID: 38351322*; Yi Wang et al., Integrated Genomic and Transcriptomic Analysis Reveals the Activation of PI3K Signaling Pathway in HPV-Independent Cervical Cancers. British Journal of Cancer, 130,6: 987–1000, 2024;

PMID: 38253702). Our results revealed that loss of NUMB significantly correlates with downmodulation of NOTCH signaling. Similar results were obtained in the GSEA of the transcriptomes of BBN-NUMB-KO *vs.* BBN-WT mouse tumors (see Figure 1a provided for the Reviewer's perusal).
b)In situ IHC analysis of BBN-NUMB-KO *vs.* BBN-WT mouse tumor transplants using antibodies directed against the active cleaved forms of NOTCH1 and NOTCH2 receptors revealed a significant downregulation in the overall percentage and intensity of tumor cells displaying intranuclear accumulation of active NOTCH, with similar results for both NOTCH receptors, in the NUMB-KO condition compared to WT (see Figures 1b,c provided for the Reviewer's perusal).
c) GSEA of the transcriptomes of NMIBC patients of the UROMOL cohort, stratified by the $NUMB^{LESS}$ signature, revealed a significant downregulation of a NOTCH transcriptional signature ("signaling by NOTCH signature", by Reactome) in patients classified as $NUMB^{LESS}$-like (reflecting a NUMB-dysfunctional status) *vs.* $NUMB^{LESS}$-not-like patients (i.e., with a proficient NUMB status) (see Figure 1d provided for the Reviewer's perusal).

We did not include these results in the original version of the manuscript because of their correlative nature. However, we are willing to include these results in the revised manuscript if the Reviewer believes that this will improve the overall quality of the study.
Mechanistically, the role of the NOTCH pathway in the homeostasis of different tissues appears to be highly context dependent, where the expression of different combinations of NOTCH receptors and cognate ligands, in space and time, largely account for the complexity of NOTCH signaling regulation and its context dependency [reviewed in Bray, S. J. *Notch signaling in context*. Nat. Rev. Mol. Cell Biol. 17, 722–735, 2016. DOI: 10.1038/nrm.2016.94)]. This complexity also depends on the crosstalk and dynamic interplay of NOTCH signaling with other pathways, such as the YAP signaling pathway, which can influence NOTCH activation *vs.* downregulation in different contexts [reviewed in C. Engel-Pizcueta and C. Pujades. *Interplay Between Notch and YAP/TAZ Pathways in the Regulation of Cell Fate During Embryo Development*. Frontiers in Cell and Developmental Biology, 9: 711531, 2021. DOI:10.3389/fcell.2021.711531]. For instance, YAP has been reported to inhibit NOTCH signaling in basal layers (A. Totaro et al., *Crosstalk between YAP/TAZ and Notch signaling*. Trends Cell Biol., 28(7): 560–573, 2018. DOI:10.1016/j.tcb.2018.03.001). In the context of the basal layer of the bladder mucosa, it is tempting to speculate that loss of NUMB-mediated inhibition of YAP signaling might lead to NOTCH inactivation; a hypothesis that would warrant an entire line of investigation.

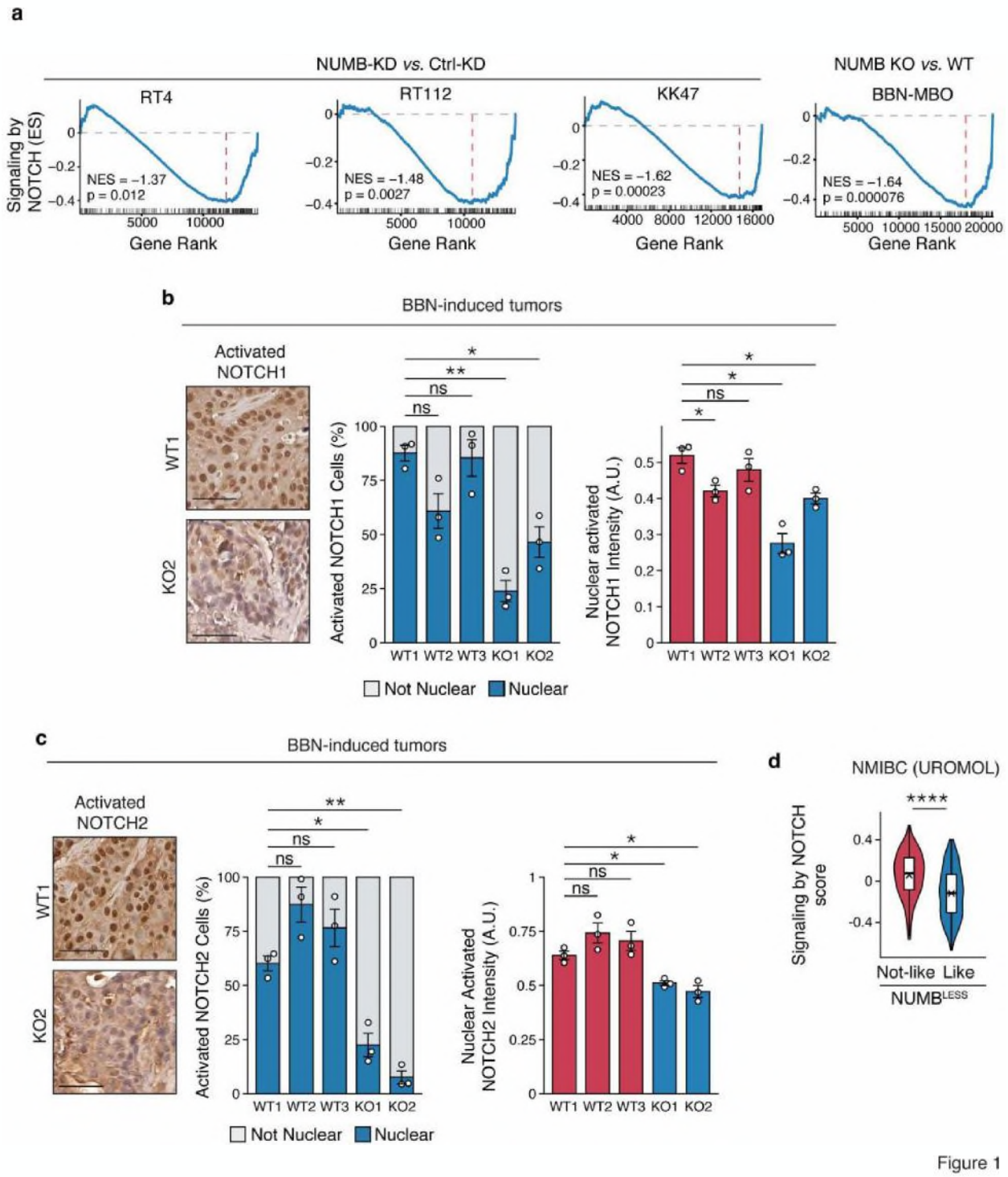

Figure 1

Figure 1. a. *GSEA showing enrichment of the Reactome "Signaling by NOTCH" gene signature in the three NUMB^{High} cell lines, RT4, RT112 and KK47, silenced or not for NUMB (NUMB-KD vs. Ctr-KD) (left, n=2) and in NUMB-KO vs. WT-BBN-MBO (right, n=3). ES, Enrichment Score; NES, Normalized Enrichment Score; p, permutation test p-value.* ***b,c.*** *Primary infiltrating BCa induced by 20 weeks of BBN treatment in WT and NUMB-KO mice were excised and examined by IHC for activated NOTCH1 (AB8925, Abcam; 1:350) (b) or activated NOTCH2 (07-1234, Merck; 1:100) (c). Left, Representative IHC images of BBN-NUMB-KO and BBN-WT tumors. Bars, 50 sum. Right, Quantification of the % of cells with nuclear activated NOTCH1 or NOTCH2 and mean nuclear activated NOTCH1 and NOTCH2 intensity. Data are expressed as mean ± SEM, n=3 fields for each sample. **, p<0.01; *, p<0.05; ns, not significant, by FDR adjusted pairwise Welch's t-test.* ***d.*** *Box-violin plot showing the "Signaling by NOTCH" score in the NUMB^{LESS}-Like and NUMB^{LESS}-Not-Like groups of the 535 NMIBC of the UROMOL cohort. ****, p<0.0001 by Welch's t-test.*

5) *What are the putative causes of NUMB loss of expression during BCa progression and in a subset of patients? Are there NUMB genomic alterations? This could be easily checked by looking at published datasets. Putative epigenetic mechanisms? The authors also show that Numb is spontaneously lost upon tumor progression (Fig.2, wild type mice) which may imply that this is a consequence of tumor progression rather than a cause (or both, difficult to discriminate in patient cohorts).*

R. Acknowledged. We agree with the Reviewer that it is difficult to determine through the analysis of patient cohorts whether NUMB loss-of-function is a primary event that promotes tumor formation or a secondary lesion selected during bladder tumorigenesis due to oncogenic insults, as shown in our BBN carcinogenesis studies. Additionally, it is challenging to ascertain whether NUMB loss-of-function, occurring as a primary or secondary event, involves different or similar upstream mechanisms.

Following the Reviewer's suggestion, we interrogated the TGCA dataset in search for possible genetic alterations that might account for loss of NUMB protein expression in human BCa patients. This analysis revealed an extremely low frequency of mutations or deletions in the *NUMB* gene (approximately 2.5%), with the most frequent alteration being gene amplification. These observations argue against genetic lesions at the *NUMB* locus being the primary or secondary mechanism responsible for loss of NUMB protein expression in BCa (see Figure 2a provided for the Reviewer's perusal). Remarkably, similar findings were observed in the TGCA dataset when we analyzed genetic *NUMB* alterations in breast cancer. In this case, the overall rate of genetic lesions at the *NUMB* locus was only 1.2% (see Figure 2b provided for the Reviewer's perusal). Together, these results suggest that genetic lesions at the *NUMB* gene locus are unlikely to be the primary or secondary cause of NUMB dysfunction in both the bladder and mammary gland.

In this regard, we would like to bring to the Reviewer's attention the results of a comprehensive study in breast cancer. In this work, we compiled a list of potential alterations underpinning NUMB dysfunction in breast cancer by integrating knowledge obtained from previous studies in the lab (S. Pece et al.,J. Cell Biol., 167,2: 215–21, 2004. *PMID. 15492044*; I.N. Colaluca et al., J. Cell Biol., 217,2: 745–62, 2018. *PMID. 29269425*; M.G. Filippone et al., J. Cell Biol., 221,12, 2022. *PMID.29269425*), with new findings highlighting specific upstream molecular circuitries involved in NUMB hyperdegradation (see Figure 2c provided for the Reviewer's perusal). These results, which are included in a separate manuscript submitted for publication, suggest that various alterations, including post-translational modifications of the NUMB protein (such as inactivating phosphorylation, and exaggerated ubiquitination leading to aberrant proteasomal degradation) and isoform-specific transcriptional regulation, are the predominant mechanisms downmodulating NUMB expression (see legend to Figure 2c provided for the Reviewer's perusal and references herein).

Based on these data, we believe that the molecular mechanisms responsible for NUMB dysfunction in BCa likely comprise diverse mechanisms, as identified in breast cancer. The characterization of these mechanisms in BCa would require long-lasting extensive investigations that extend beyond the scope of this study. We believe this work would be better presented in a more focused study.

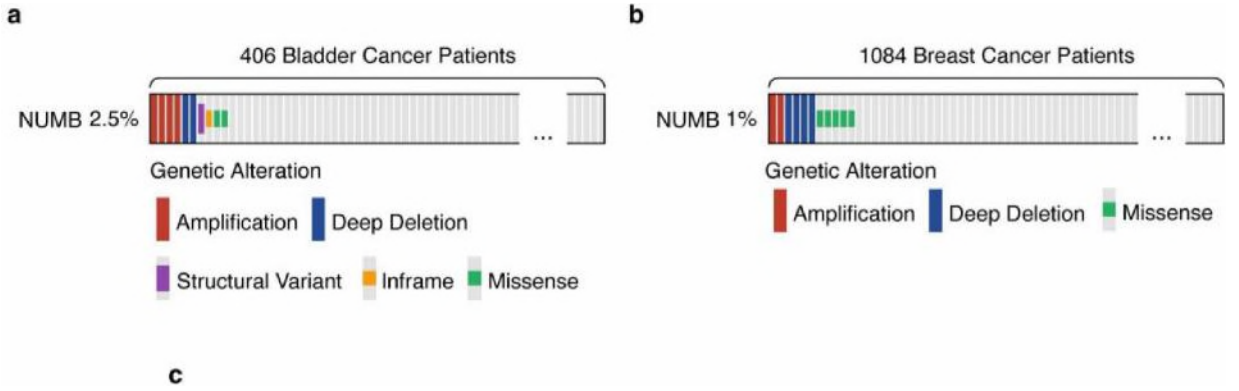

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Figure 2

Figure. 2. Molecular mechanisms responsible for NUMB dysfunction in cancer. a. *Frequency of genetic alterations of the NUMB gene in BCa in the TCGA dataset.* ***b.*** *Frequency of genetic alterations of the NUMB gene in breast cancer in the TCGA dataset.* ***c.*** *Mechanisms underlying NUMB alterations in breast cancer. The pie chart shows the relative contribution of the various NUMB loss-of-function mechanisms in breast cancer. The most frequent alteration is decreased protein expression due to exaggerated ubiquitination and ensuing protesomal hyperdegradation (~36%), (S. Pece et al. J. Cell Biol. 2004. PMID: 15492044 and submitted paper). Data for "splicing alterations with loss of Exon 3" and "protein inactivation by hyperphosphorylation" are from I.N. Colaluca et al. (J. Cell Biol., 217,2: 745–62, 2018. PMID: 292694259) and M.G. Filippone et al. (J. Cell Biol., 221,12, 2022. PMID:29269425), respectively. We note that the category "No alteration evidenced" likely comprises miRNA-based mechanisms, which are currently under investigation in our lab.*

Minor comments:

- Page 8. "In mouse and human" while mouse experiments have not been introduced in the text yet

R. Agree. The entire sentence containing the word 'mouse' that was present in the old version has been eliminated in the text of the revised manuscript.

- Double check nomenclature to refer genes vs. proteins in mouse vs. human (small vs. capital, italic vs normal).

R. Agree. We have checked the manuscript and modified the nomenclature according to international standard guidelines (mouse: www.informatics.jax.org; human: https://www.genenames.org):

i) for human, gene symbols italicized and all letters in uppercase, protein symbols not italicized and all letters in uppercase;

) for mouse, gene symbols italicized, with only the first letter in uppercase, protein symbols not italicized, and all letters in uppercase.

- The "Real-life" in not necessary when referring to BCa patients

R. Agree. We have amended the text as indicated.

Reviewer #2

This a very interested and well crafted manuscript on a subject that is both highly significant and understudied. The manuscript by Tucci et al investigates the role of Numb in the transition from non-muscle invasive (NMIBC) to muscle invasive bladder cancer (MIBC). First they show that numb expression is associated with disease outcome in patients, and link this to YAP signaling. They then perform functional studies in mouse models and organoids to investigate these activities. They propose - and present data to support - that this provides a therapeutic opportunity for treating this group of patients that have few treatment options. Overall, the work is of high quality and the manuscript well-written and well presented. This review particularly appreciates the careful analyses of the organoid models. I have only a few comments to further improve this fine study.

R. We thank Reviewer #2 for the appreciative words on our study and for the suggestions provided, which helped us to produce an improved version of our manuscript.

1) The rationale for studying Numb in the first place needs to be introduced -- ideally in the introduction and I think also in the first paragraph of the results.
R. Agree. We have now inserted in the Introduction (see page 4, lanes 58-62), as well as at the beginning of the Results (see page 6, lane 79-81) and Discussion (see page 23, lanes 510-515) of the revised manuscript a better explanation of the rationale for investigating the potential relevance of NUMB dysfunction to BCa pathogenesis.

2) The patient analyses regarding NUMB expression and YAP activity is very important and this reviewer thinks could be highlighted more effectively in the Main text. I also think this is a bit confusing to divide into 3 separate sections of the results. I would suggest to move some of the data from the Supplement to the main body and/or split to 2 figures. In particular, it would be beneficial to see outcome data for the YAP - currently the data in the main body only shows the relation of Numb and YAP but not the relevance for patient outcome.
3) Related to the comment above - the gene signature data should be part of the main body as this is most directly translatable.
R. Agree. We are most grateful to this Reviewer for the number of suggestions and detailed indications provided in these two points. Based on these suggestions, we have performed a series of new analyses and reorganized the clinical data to better highlight the potential clinical impact of our study. We have now assembled all the clinical data for NUMB and YAP into two new figures in the main text (see Figures 1 and 2 and Supplementary Figures 1 and 3 of the revised manuscript), as detailed below:
A) Figure 1 now contains all data establishing the relevance of NUMB dysfunction to BCa:
i) The analysis of the prognostic value of NUMB, assessed by IHC, in the IEO MIBC and NMIBC patient cohorts (see new Figure 1b,c which are the same as the old Figure 1).
ii) A better representation of the derivation and composition of the $NUMB^{LESS}$ signature, with a detailed description of the downregulated and upregulated gene sets following NUMB silencing in the three NMIBC NUMB-proficient human cell lines (RT4, RT112, and KK47) (see new Figure 1f, left panel);
iii)The clustering analysis of the different $NUMB^{LOW}$ (5637, CLS439 and HT1376) *vs.* $NUMB^{HIGH}$ (KK47, RT112, RT4) and NUMB-KD *vs.* Ctrl-KD KK47, RT112 and RT4 cells, using the $NUMB^{LESS}$ signature (see Figure 1f, right panel, previously old Extended Data Figure 2b).
iv)The clustering analysis of the 535 NMIBC patients of the UROMOL cohort using the $NUMB^{LESS}$ signature (see Figure 1g, previously old Extended Data Figure 2c).
v) The prognostic stratification for risk of muscle invasion using the $NUMB^{LESS}$ signature in NMIBC patients of the UROMOL cohort (see new Figure 1h, previously old Figure 1), alongside a

multivariable analysis of progression-free survival of these patients adjusted for clinicopathological parameters (see Figure 1i, previously old Extended Data Figure 2d).

B) Figure 2 now contains all data establishing the relevance of the connection between NUMB loss and YAP/EMT activation to NMIBC patients (see also our response to Reviewer #1, point 1), including patient outcome data for YAP, according to this Reviewer's request:
i) The results from transcriptomic profiling studies in $NUMB^{LOW}$ (5637, CLS439 and HT1376) *vs.* $NUMB^{HIGH}$ (KK47, RT112, RT4) and NUMB-KD *vs.* Ctrl-KD KK47, RT112 and RT4 cells, which highlight YAP activation and subsequent EMT activation as molecular hallmarks of the NUMB-defective condition (see new Figures 2a-e of the revised manuscript).
i) Patient outcome data for YAP in the 535-NMIBC UROMOL cohort clusterized by the 22-gene YAP signature (see new Suppl. Figure 3b, previously Extended Data Figure 1c). This analysis demonstrates that an active YAP status predicts increased rate of muscle-invasion progression in a multivariable analysis adjusted for standard clinicopathological parameters (see new Figure 2f, left panel and new Suppl. Figure 3c).
ii) Evidence in the UROMOL cohort of the association between active YAP status, assessed by the 22-gene YAP signature and a NUMB-defective condition, as assessed by the $NUMB^{LESS}$ signature (see new Figure 2g, left panel, previously old Figure 1i), alongside evidence of the association between active YAP/NUMB dysfunction and EMT activation (see new Figure 2f,g, right panels).
iii) A new set of immunofluorescence (IF) images, with relative quantitation, obtained by *in situ* analysis of FFPE samples, showing the distribution of YAP and its downstream transcriptional target, CYR61, in NMIBC $NUMB^{HIGH}$ *vs.* $NUMB^{LOW}$ patients (see new Figure 2h). This figure replaces the old Figure 1j and also addresses point 5 (see below) from this Reviewer.

4) *What is the relationship of the current gene signature to previous gene signatures for bladder cancer outcome - this information could be included as supplementary. Here I am thinking to show if there is overlap of the genes in the signatures and/or comparison of their strength -- so, are they complementary and add value to each other OR very similar to each other. This reviewer realizes that most readers of Nature Comm will be interested in the mechanistic figures that follow, but the relationship to bladder cancer outcome is very important and should be better highlighted.*
R. Agree. This useful suggestion prompted a series of clinical analyses to benchmark the $NUMB_{LESS}$ signature against other BCa-specific transcriptomic signatures described in the literature. The new results, summarized below (see new Suppl. Figure 2 and text on page 9, lanes 153-171 of the revised manuscript), greatly strengthen the potential clinical impact of our study, for which we are grateful:
i) We show that there is very limited overlap between the $NUMB^{LESS}$ signature and other BCa signatures, with only 4 genes in common, two of which (HMOX1 and IL1B) exhibit an opposite direction of regulation, in association with prognosis, in the $NUMB^{LESS}$ signature compared with the other signatures (see new Suppl. Figure 2a).
ii)The $NUMB^{LESS}$ signature ranks as the third most potent signature providing statistically significant prognostic information over the standard clinicopathological parameters in a multivariable analysis. In this analysis, the different signatures were individually challenged against standard clinical variables for prediction of risk of muscle invasion in the UROMOL NMIBC patient cohort (see new Suppl. Figure 2b and Methods on page 42, lanes 999-1006 of the revised manuscript).
iv) We performed a Lasso penalized Cox regression analysis to assess the redundancy between the various signatures, as well as the clinicopathological variables, in providing prognostic information. Strikingly, the $NUMB^{LESS}$ signature not only retained its prognostic value against all the other signatures, but also emerged as the signature with the highest prediction coefficient among the few other signatures that retained a significant prognostic value after multivariable adjustment for all the other signatures and clinical parameters (see new Suppl. Figure 2c).

Together, these results indicate that *i*) the prognostic information provided by the $NUMB_{LESS}$ signature is most likely derived from molecular and phenotypic features inherent to BCa biology,

which differ from those associated with other signatures; *ii*) the NUMB^{LESS} signature, whether used alone or in combination with other signatures, has the potential to be developed into a clinical test for risk stratification of NMIBC patients.

5) *Panel 1j - is not very compelling and could be improved.*
R. Agree. The direct assessment by IF of active YAP intranuclear accumulation in FFPE samples from NUMB^{HIGH} *vs.* NUMB^{LOW} NMIBC patients, shown in the old panel 1j, was aimed to provide further evidence of the relevance to human BCa of the NUMB-YAP connection identified in human cell lines and already validated in the UROMOL patient cohort (see new Figure 2g, old Figure 1i).

We have now included in the new Figure 2h of the revised manuscript (old Figure 1j) more representative images for the YAP staining. To further corroborate these results, we have now also analyzed the distribution of a direct YAP transcriptional target, CYR61, based on evidence that:
i) CYR61 is included in the set of upregulated genes in the NUMB^{LESS} signature (CCN1 in the left panel of the new Figure 1f).
ii)CYR61 is upregulated in both the 22-gene YAP signature and the EMT activation signature used in our study (see new Suppl. Figure 12a).
) CYR61 was validated as the functional link, together with CTGF, between YAP and EMT activation associated with the NUMB-deficient condition in the human RT4 NMIBC cell line and mouse BBN-tumor model (see new Suppl. Figure 12b-g).

6) *For the mouse model - if this reviewer understands correctly (from the methods), the model has a flexed allele of Numb crossed with a CK5-Cre allele - if so, this will delete numb in all CK5 expressing cells (which are in many tissues). How do the authors know that they effects they are seeing are cell-autonomous to the bladder. If they do not know, they should add a caveat so as not to be misleading. This is especially important for the data in Fig. 2 showing that Numb alone promote a pre-cancerous and even cancerous phenotype in bladder. (very few genes do so).*
R. We acknowledge this relevant point and refer this Reviewer to our response to a similar point raised by Reviewer #1 (point 2). We provide explanations and experimental data supporting the concept that it is highly unlikely that the histopathological alterations and the reduced survival observed in the CK5-NUMB-KO model are due to homeostatic alterations in tissues other than the bladder.

We also agree with this Reviewer that the CK5-NUMB-KO model is a unique model of aggressive bladder tumorigenesis. We plan to make this model available to the scientific community for future studies on BCa biology. However, we believe that the potent tumor-promoting effect of NUMB loss in the bladder mucosa is not unexpected based on the following considerations:
i) NUMB acts as an upstream regulator of several tumor suppressor and oncogenic pathways, whose simultaneous dysfunction likely synergistically cooperates to promote malignant transformation of the bladder urothelium. For instance, NUMB positively regulates the tumor suppressor activity of p53 (Colaluca IN et al. *NUMB controls p53 tumour suppressor activity*. Nature 2008, 451:76-80; *PMID: 18172499*). p53 dysfunction in the basal urothelial layer is a well-characterized molecular event that underlies neoplastic transformation of the bladder mucosa (Park S. et al. *Novel Mouse Models of Bladder Cancer Identify a Prognostic Signature Associated with Risk of Disease* Progression. Cancer Res 2021, 81:5161-5175; *PMID: 34470779*). Concomitantly, as demonstrated in this study, NUMB dysfunction results in YAP activation, which has an established pathogenetic role in bladder tumorigenesis (S. W. Baek, et al., *YAP1 activation is associated with the progression and response to immunotherapy of non-muscle invasive bladder cancer*. EBioMedicine, 81:104092, 2022. *PMID: 35665684*; M. K. Gill, et al., *A feed forward loop enforces YAP/TAZ signaling during tumorigenesis.* Nat Commun, 9:1,3510, 2018. *PMID 35665684*; J. Y. Liu, et al., O*verexpression of YAP 1 contributes to progressive features and poor prognosis of human urothelial carcinoma of the bladder*. BMC Cancer, 13:349, 2013. *PMID: 238704129*). We also note that NUMB dysfunction results in downregulation of NOTCH (see our findings shown in Figure 2 of this rebuttal, in response

to point 4 from Reviewer #1), which has been reported to play a tumor suppressor role in the bladder urothelium.
7) It would be beneficial to name the pathologist making the evaluation of the phenotype (in the methods).
R. Agree. The name of the pathologists that have performed histological evaluations have been now included in the Materials and Methods of the revised version (see page 30, lane 693 and 706).

8) I do not see methods for how they made the organoids - including the age, sex, how they are selecting the cells (sorting?) etc. This is a serious omission that needs to be corrected in the methods.
R. Agree. We thank the Reviewer for highlighting this omission. We have now provided this information in the revised Materials and Methods (see page 32-33, lanes 750-763).

9) Along these lines, I also do not see whether they are looking at both males and females and whether there are differences. This is very important and should be highlighted throughout.
R. Agree. We refer this Reviewer to our response to a similar point raised by Reviewer #1 (point 3), where we provide a detailed explanation for the rationale of using male mice.

10) Some of the conclusions are stated too strongly and should be toned down. This would not detract at all from the paper. For example (but there are others) -- this statement -- "Thus, Numb status could guide clinical decision-making between conservative vs. more aggressive therapies" is much too strongly stated.
R. Agree. We have revised the text following the Reviewer's suggestion to down tone the conclusions relating to the use of NUMB as a clinical biomarker, starting from the sentence highlighted by the Reviewer (see page 7, lanes 105-107 of the revised manuscript). In this regard, we more accurately discuss the clinical potential of NUMB in light of the new data generated by the comparison of the $NUMB^{LESS}$ signature with other existing signatures (see point 4), suggesting that NUMB status might be informative in prognostic nomograms for BCa in combination with other existing molecular signatures and clinicopathological parameters (see page 9, lanes 168-171 in the Results, and page 26, lanes 597-604 in the Discussion of the revised manuscript).

0) Why did they limit analysis of the drug to BBN organoids only? I dont think I missed the data for the non-BBN organoids in the supplement.
R. Agree. Admittedly, in the original manuscript, we limited our pharmacological inhibition studies on non-BBN organoids (i.e. from BBN-naïve NUMB-KO and WT mice) to the use of the YAP inhibitor, verteporfin. The aim of these studies was to assess the relevance of YAP activation to the aberrant morphological traits associated with absence of NUMB in this model. These results were already shown in the old Figure 3b,c, and are now shown in the new Figure 4d,f of the revised manuscript, with the addition of data from new experiments in which verteporfin was a control for the ROCK inhibitor in the treatment of non-BBN organoids, as described below.

Prompted by this Reviewer's point, we have now also analyzed in non-BBN organoids the effects of pharmacological inhibition of ROCK. These new findings show that:
i) ROCK inhibition profoundly decreases the frequency of aberrant proliferative and invasive morphological phenotypes in non-BBN NUMB-KO organoids, with no substantial effects on non-BBN WT organoids (see new Suppl. Figures 9f-h).
ii) ROCK inhibition results in impaired YAP transcription and EMT activation (see Suppl. Figures 9i,j).

Together, these results indicate that the phenotypic and molecular differences observed in non-BBN NUMB-KO *vs*. WT organoids depend on the ROCK/YAP axis, recapitulating the effects observed in BBN organoids. Consistently, we also show now that EMT activation is also a distinctive molecular hallmark of non-BBN NUMB-KO *vs.* WT organoids, and can be reverted by

pharmacological inhibition of YAP (see new Figure 4c) and ROCK (see new Suppl. Figure 9j). Furthermore, these findings address an important point raised by Reviewer #3 (point 4) questioning the connection between YAP signaling deregulation and EMT activation in association with a NUMB-deficient status.

12) I think the drug has to be better characterized for its specificity. I see knock-down data in the supplement - but I think this could be more explicitly shown.
R. Agree. We have now moved in the revised manuscript the set of results that were shown in the old Extended Data Figures 5b-e to the main figures (see new Figure 5f-i). These results show that YAP knockdown fully phenocopies the inhibitory effects of the YAP inhibitor, verteporfin, in NUMB-KO *vs.* WT BBN-tumor organoids. In the same model, we also tested the specificity of the RHOA inhibitor, C3 transferase, and the RAC inhibitor, NSC23766, using dominant-negative mutants of RHOA and RAC as controls (these results, shown originally in the old Figures 4e-g and Extended Data Figures 8a,b,d, are now shown in the new Figures 6e-g and new Suppl. Figures 9a,b,d).

13) I find this a bit confusing to conclude the paper with the data in Fig. 7 - which is disconnected to Fig. 1. I wonder if the authors could find a better way to coordinate these findings for improved clarity.
R. Agree. Following the Reviewer's suggestion, we better illustrate the pathogenetic circuitry underlying the association between NUMB dysfunction and aggressive BCa biology, and how this might be germane to the aggressive outcome of the NMIBC disease observed in our clinical studies. We have included a new schematic representation (see new Figure 9c) depicting the dependency of the activation state of the RHOA/ROCK-Hippo/YAP pathway on NUMB status and the progression to muscle invasion when this circuitry is functionally upregulated as a consequence of NUMB dysfunction.
We also indicate the potential sites of the RHOA-ROCK-YAP circuitry that might represent actionable points for therapeutic intervention to prevent progression to muscle-invasive disease, likely through the reversion of EMT and inhibition of proliferative/migratory phenotypes.

14) Again, while this Reviewer understands that most readers will be most interested in the mechanistic relationships, the value to patients with NMIBC is very important not really highlighted in the discussion. I think this is a missed opportunity and a few sentences would help a lot.
R. Agree. We are most grateful to the Reviewer for this insightful suggestion. We have highlighted better the potential clinical relevance of our findings in light of the unmet clinical needs of the naturally occurring NMIBC disease. In the Discussion, we discuss the potential clinical value of NUMB as a biomarker to stratify NMIBC patients by risk of progression and response to novel targeted anti-RHOA/ROCK/YAP therapies (see page 26, lanes 597-604 and lanes 630-635).

Reviewer #3

In this manuscript, authors found loss of Numb expression correlates with worse overall survival in post-cystectomy muscle-invasive bladder cancer (MIBC) patients and increased risk of MIBC progression in non-muscle-invasive bladder cancer (NMIBC) patients. Using Numb knockout transgenic mouse model and BBN carcinogen treatment model, authors further demonstrated the tumor suppressor role of Numb in bladder cancer. Using 3D-Matrigel organoid culture model, authors found that Numb loss heightens the proliferative and invasive potential of both mouse and human bladder cancer (BCa) cells. Integrative transcriptomic and functional analyses revealed that downregulation of the canonical Hippo pathway, resulting in enhanced YAP transcriptional activity, underlies the biological aggressiveness of Numb-deficient BCa. These molecular events are dependent on the activation of RhoA/ROCK signaling subsequent to Numb loss. Thus, a dysfunctional Numb–RhoA/ROCK–Hippo/YAP regulatory network is at play in aggressive Numb-deficient BCa and represents a therapeutic vulnerability.

The manuscript was well-written, and findings are interesting.
R. We are most thankful to Reviewer #3 for the appreciative words on our study and for the suggestions provided. This Reviewer raised several highly relevant points that we address below *(the points are reproduced verbatim, maintaining the reviewer's original numbering for convenience)*:

Major points:
1) The immunoblot of Numb sometimes showed two bands, sometimes showed one band. Can authors explain this discrepancy?
R. Agree. NUMB is expressed as 4 isoforms (p72, p71, p66, p65) that differ in the presence of two alternatively spliced exons: exon 3, comprised of 33 nucleotides (11 amino acids), in the N-terminal PTB domain; exon 9, comprised of 144 nucleotides (48 amino acids), in the C-terminal region (Dho, SE et al., *Characterization of four mammalian numb protein isoforms. Identification of cytoplasmic and membrane associated variants of the phosphotyrosine binding domain*. J. Biol. Chem. (1999). DOI:10.1074/jbc.274.46.33097; Verdi, J. M. et al. *Distinct human NUMB isoforms regulate differentiation vs. proliferation in the neuronal lineage*. Proc. Natl. Acad. Sci. U. S. A. (1999). DOI:10.1073/pnas.96.18.10472).

In immunoblots, the four NUMB isoforms appear as a doublet, with an upper band containing isoforms p72 and p71, and a lower band containing isoforms p66 and p65, which run very close to each under standard SDS-PAGE conditions.

It is also important to note that the expression of the four NUMB isoforms varies depending on cell context. This is evident comparing the expression levels of the upper and lower bands of the NUMB doublet in NUMB-proficient and NUMB-deficient human cell lines (see, for instance, the blots of $NUMB^{HIGH}$ *vs.* $NUMB^{LOW}$ bladder cell lines in the new Figure 1e, previously old Figure 1d; see also Figure 3 provided for Reviewer's perusal below for an immediate comparison of the NUMB isoform expression pattern across the different human BCa cell lines).

Importantly, mouse *vs.* human variations in the NUMB expression pattern also exist, as is evident from the immunoblots of mouse cells where the upper band is typically expressed at lower levels compared to the lower band (see, for instance, the blots of BBN-WT cells in the new Figures 5f, 6b, 7a and Supplementary Figures 7e, 9c, previously shown in the old Figure 4b, 5a, and Extended Data Figure 5b, 6e, 8c; see also Figure 3 provided for Reviewer's perusal). Importantly, similar human *vs.* mouse differences in the NUMB expression pattern were observed in the mammary gland in our previous studies (Tosoni et al., JCB 2015; DOI: 10.1083/jcb.201505037; Colaluca et al., JCB 2018 DOI: 10.1083/jcb.201709092; Filippone et al., JCB 2022; DOI: 10.1083/jcb.202112001).

For an immediate comparison of the patterns of NUMB expression in $NUMB^{HIGH}$ *vs*. $NUMB^{LOW}$ human BCa cell lines, and in BBN mouse cells, we provide here for the Reviewer's perusal a comprehensive representation (see Figure 3) of all the immunoblot panels included in the revised

version of the manuscript (*numbered according their order of appearance in the new Figures and Supplementary Figures*). Here, we also show a shorter exposure of NUMB expression in Figure 6b. This shorter exposure has been used in the new Figure 6b of the revised manuscript to replace the longer exposure previously shown in the old Figure 4b, as it appears more consistent with the pattern of NUMB isoform expression shown in all the other BBN mouse experiments.

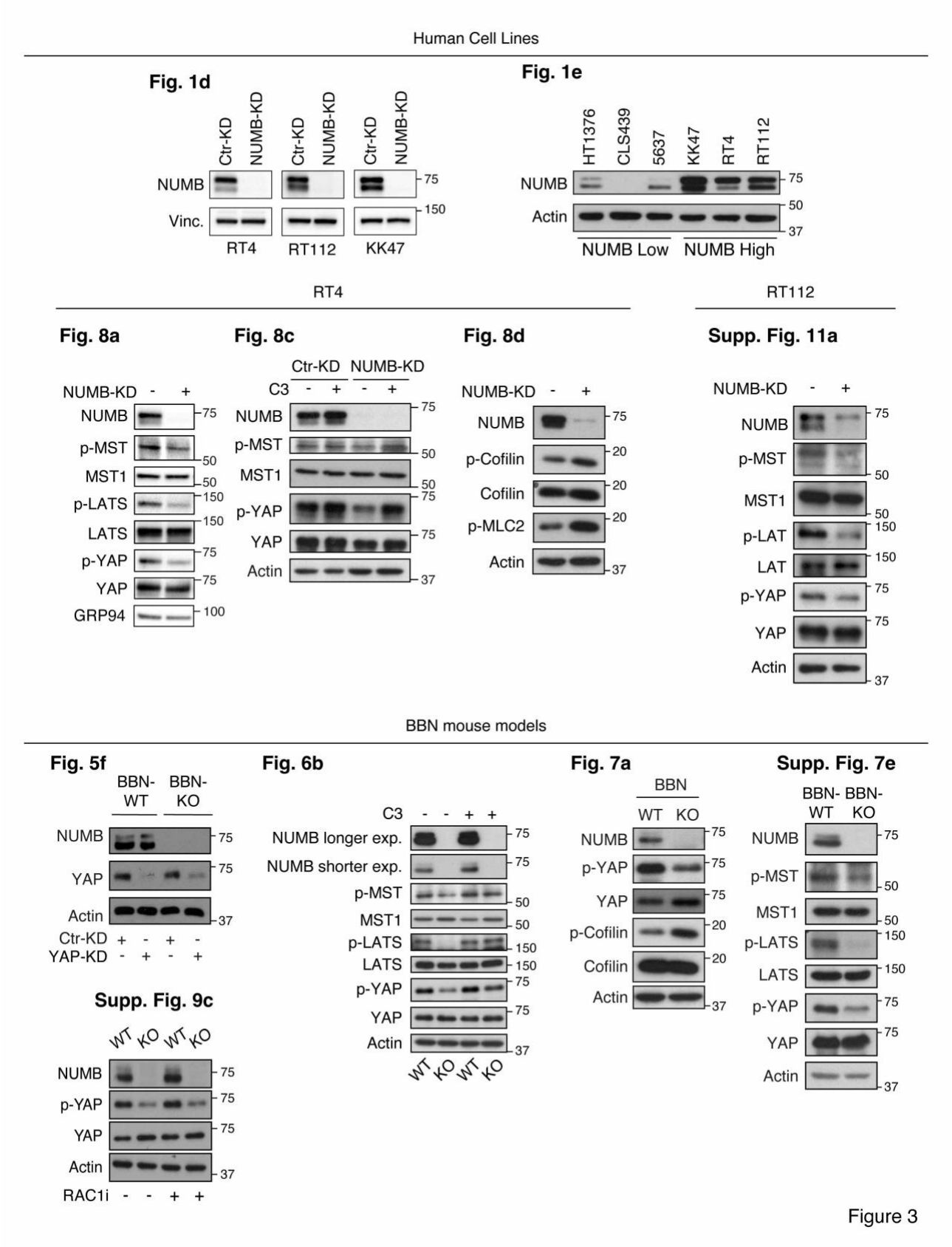

Figure 3. *Comprehensive view of immunoblots showing the pattern of NUMB isoform expression expression if the various human BCa cell lines and in BBN mouse cells.*

2) It is unclear the time frame of Bca tumor onset in Numb-knockout mice.
R. We acknowledge this Reviewer's point. To address this point, we have now included a new figure (see Suppl. Figure 4d in the revised manuscript) describing in detail the timeframe of appearance of the histological alterations in the bladder mucosa in NUMB-KO *vs.* WT mice.

3) Figure 3a-b, why the WT 3D organoid structures were so different? Figure 3b showed multi-acini structure in Numb-KO, what is frequency of this phenotype?
R. We acknowledge this Reviewer's point. The Reviewer appropriately noted that the morphology of WT organoids shown in the IF images of the old Figure 3a (now Figure 4a in the revised manuscript) is different from the WT organoid morphology shown in the brightfield images in the old Figure 3b (Figure 4b in the revised manuscript). However, this discrepancy is only apparent and is due to the stepwise procedure required to prepare organoid samples for IF analysis, which includes enzymatic digestion of the 3D-Matrigel matrix to yield purified organoids, followed by a centrifugation step to enrich and concentrate the organoids in a small pellet suitable for subsequent paraffin inclusion. This procedure leads to a profound alteration of the typical WT organoid morphology and results in the appearance of organoid aggregates in IF images, which contrast with the images obtained by direct brightfield microscopy of whole 3D-Matrigel plates. These brightfield images depict the 'real' appearance of organoids as isolated, clonally derived structures in 3D-Matrigel cultures. To clarify this point, we have included in the Material and Methods of the revised manuscript a more accurate description of the stepwise procedure required for the preparation of WT organoid samples for IF analysis (see page 36, lanes 856-865). We are grateful to the Reviewer for prompting us to clarify this apparent discrepancy.

We also specifically address the Reviewer's question about the frequency of the multi-acinar phenotype in WT *vs.* NUMB-KO 3D-Matrigel organoids. To do this, we include in the revised manuscript a quantitation of the morphotypes observed in the respective conditions from the experiments depicted in the old Figure 3b (see new Figures 4d-f). This analysis clearly shows that the aberrant multi-acinar morphology is a phenotype selectively associated with the NUMB-KO condition, which never appears in WT organoids.

4) The relationship between EMT and VP mentioned but was not further discussed.
R. Agree. To address this point, we have now included in several parts of the revised manuscript, a more in-depth discussion of the correlation between YAP activation and EMT, based on experiments of EMT reversion by verteporfin (VP) (see page 10, lane 183-192; page 5, lane 75; page 14, lanes, 293-300; page 15-16, lanes 328-335). In more general terms, we have better highlighted the relevance of the YAP-dependent EMT activation, through RHOA/ROCK, to the aggressive biology of NUMB-deficient BCa (see page 2, lane 17; page 5, lane 75; pages 10-11, lanes 193-213; page 16, lanes 348352; pages 19-20, lanes 420-438; pages 20-21, lanes 459-463; page 22, lanes 489-509, page 25, lanes 563-567).

The link between YAP activation and EMT was further evidenced in the following new experiments:
a) New RNAseq-based analysis of human BCa cell lines and non-BBN mouse organoids treated or not with verteporfin. This analysis demonstrated the dependency of the NUMB loss-associated EMT activation on YAP signaling (see new Figures 2e and 4c, respectively in the revised manuscript), which we previously evidenced in the BBN-organoid model (old Extended Data Figure 5a, now Figure 5e of the revised version).
b) Studies in human BCa cell lines, as well as in mouse non-BBN and BBN-tumor organoids, showing that pharmacological targeting of the RHOA-ROCK axis results in EMT inhibition (see new Figures 7h and 8f, and new Suppl. Figures 9j and 11e).
c) Genetic silencing of two YAP transcriptional targets, CYR61 and CTGF, previously reported to be EMT activators downstream of YAP (I. Haque et al., *Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis.* Mol

Cancer (2011); PMID: 21232118; S. Sonnylal, et al. *Connective tissue growth factor causes EMT-like cell fate changes in vivo and in vitro*. J Cell Sci (2013). PMID: 23525012), results in the reversion of NUMB loss-associated EMT induction in human NUMB-KD RT4 cells and mouse BBN-NUMB-KO-MBOs (see new Suppl. Figure 12). This finding establishes a direct functional correlation between specific YAP transcriptional targets and activation of the EMT program, which addresses another highly relevant point raised by this Reviewer (see minor point 4, below).

5) *The study is not clear about the specific regulatory mechanisms of NUMB, RhoA/ROCK/YAP signaling pathways. Figure 5, regarding the activation of the Hippo pathway by Numb through the inhibition of the RhoA/ROCK signaling pathway, which in turn affects the transcriptional activity of YAP, a process that correlates with the aggressiveness and progression of BCa. However, the details of how RhoA/ROCK signaling is specifically regulated and the role of Numb deficiency with other potential signaling pathways are not yet clear. Are further experiments planned in follow-up, such as using mass spectrometry, to reveal the direct interactions between Numb and these signaling molecules?*

R. Agree. In the Discussion of the original version of the manuscript (now slightly modified and included in the revised manuscript; see pages 25-26, lanes 568-596), we clearly state that the mechanism(s) underlying NUMB mediated regulation of the RHOA/ROCK axis remain an open question. We also presented two, not necessarily mutually exclusive, lines of thought:

i) the existence of a mechanism inherent to the endocytic/sorting function of NUMB, influencing RHOA activation through its subcellular cytoplasmic-to-plasma membrane relocation, similarly to what we previously described for RAC1 (Zobel M et al., J Cell Biol 2018; PMID: 30061108). This would be mediated through the direct interaction of NUMB with positive (GEFs) or negative (GAPs, GDIs) regulators of RHOA activity.

ii)The direct interaction of NUMB with key components of the actomyosin remodeling and/or stress fiber formation machinery, which might in turn influence the RHOA/ROCK activation state to ultimately modulate the Hippo/YAP cascade, as also previously hypothesized (see page 25-26, lanes 580-596 and references herein in the Discussion of the revised manuscript).

Notably, we have already identified through mass spectrometry studies (in agreement with the strategy suggested by this Reviewer), a variety of candidate NUMB interactors of potential functional relevance to the above hypothesized mechanisms. Therefore, while we can provide a positive answer to the Reviewer's question as to our plans to perform follow-up studies to elucidate the underlying mechanisms regulating RHOA/ROCK activity following NUMB dysfunction, a thorough understanding of these mechanisms will take considerable time and will hopefully represent the object of a future publication.

Another important question contained in this Reviewer's point concerns the role of NUMB deficiency relative to other signaling pathways. We would like to refer this Reviewer to our response to Reviewer #1 (point 4), where we describe an extensive analysis of the NOTCH pathway in the different experimental models used in our study, showing that it is unlikely that this pathway plays a relevant pathogenetic role in NUMB-deficient BCa. Moreover, we show that the key molecular alterations associated with NUMB loss, i.e., YAP signaling and EMT activation, appear to be the major determinants of aggressive BCa phenotypes, namely invasion/migration, observed in our NUMB-deficient human and mouse models. Finally, although NUMB is known to control p53 activity (Colaluca IN et al. *NUMB controls p53 tumour suppressor activity*. Nature 2008, 451:76-80; DOI: 10.1083/jcb.201709092), the activation of YAP/EMT appears to occur independently of p53 status, since the human cell lines used in this study display different p53 status (RT4 has WT p53, RT112 and KK47 have mutated p53).

6) *Numb is a crucial determinant of cell fate, involved in regulating processes such as cell differentiation and proliferation. It may potentially influence the function of the endoplasmic reticulum and protein folding, which could in turn affect the expression and activity of GRP94, a*

molecular chaperone of the endoplasmic reticulum. It is not clear why authors used GRP94 as a loading control in Immunoblot analysis.
R. Acknowledged. In response to this Reviewer's point, we note that in our previous studies highlighting the role of NUMB dysfunction in breast cancer, we have extensively validated GRP94, interchangeably with vinculin, as a suitable loading control in immunoblot analyses comparing the NUMB-proficient *vs.* NUMB-deficient condition, in both primary mouse and patient-derived models (D. Tosoni, et al., *The Numb/p53 circuitry couples replicative self-renewal and tumor suppression in mammary epithelial cells*. J Cell Biol 2015; PMID: 26598619; D. Tosoni, et al., *Pre-clinical validation of a selective anti-cancer stem cell therapy for Numb-deficient human breast cancers*. EMBO Mol Med 2017; PMID: 28298340; M. G. Filippone, et al., *Aberrant phosphorylation inactivates Numb in breast cancer causing expansion of the stem cell pool*. J Cell Biol 2022; PMID: 36200956s).

However, to address the Reviewer' point, we have now re-analyzed by western blotting the same lysates of the old Figure 6a (now new Figure 8a), using vinculin as a loading control. This analysis, reported in Figure 4 below, provided for Reviewer's perusal, shows that vinculin faithfully recapitulates the behavior of GRP94 as a loading control.

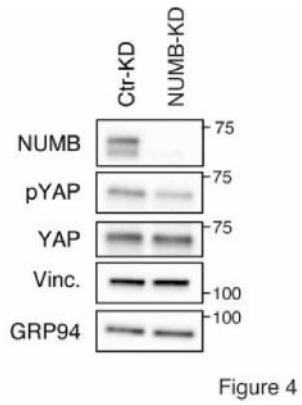

Figure 4

Figure 4. *Immunoblot analysis of the same lysates used in the old figure 6a (new figure 8a) using vinculin, alongside GRP94, as loading control for the analysis of NUMB expression in NUMB-KD vs. Ctr-KD RT4 cells.*

7) *Lack of evidence of dysfunction/activation of YAP/TAZ in bladder cancer patients.*
R. Agree. We thank the Reviewer for raising this important point, as it prompted us to investigate more in-depth the clinical value of YAP dysfunction in BCa patients. We would like to refer this Reviewer to our response to similar points raised by Reviewer #1 (point 1) and Reviewer #2 (point 2), where we describe a set of new clinical analyses obtained in the UROMOL NMIBC patient cohort. These analyses show that YAP activation *i)* behaves as a negative prognostic biomarker for risk of muscle-invasion progression (see new Figure 2f, left panel), *ii)* predicts positive response to BCG immunotherapy (see new Supplementary Figure 3d), *iii)* correlates with EMT activation (see new Figure 2f, right panel). Furthermore, we have also discussed better the evidence from the existing literature showing the relevance of YAP activation to the naturally occurring BCa.

Minor comments:

1) *Figure 1j, authors described the correlation between low expression of Numb protein and increased nuclear accumulation of YAP. However, figure 1j showed YAP positive versus YAP negative.*
R. Acknowledged. We thank the Reviewer for raising this point and making us note that the classification of cells as YAP-positive or YAP-negative in this figure was confusing. Throughout the

revised manuscript, we have now consistently classified cells as “YAP Low” or “YAP High” in all the relevant figures (see new Figures 2h, 4a and Supplementary Figure 6b,c,e, previously old Fig. 1j, 3a, and Extended Data Figure 4 b,c,e, respectively).

2)Figure 3E, the BBN-KO 3D organoid image quality needs to be improved. R.
Acknowledged. We have included a better quality image for this figure (see new Figure 5a).

3)Would it be possible to arrange Figure 2 & 5 in a landscape orientation?
R. Acknowledged. In the revised manuscript, we have arranged the new Figures in a portrait or landscape orientation, as appropriate.

4)Extended Data Fig. 5a, Supplementary Table 9, see also Supplementary Table 10 for a complete list of differentially regulated genes), which was also sensitive to VP treatment (Extended Data Fig. 5a, Supplementary Table 11). This part of the RNA-seq results is seen in the Supplementary Fig. Although GSEA analysis provides gene expression patterns, it is best to combine it with specific biological function experiments to enhance the biological significance of the results. (In this study, the authors did not design specific functional experiments to directly verify the role of YAP target genes in the EMT process).
R. Agree. We thank the Reviewer for this point which prompted us to perform a new set of molecular and functional studies (see new Supplementary Figure 12 of the revised manuscript) to investigate the mechanistic involvement of YAP transcriptional targets in the control of EMT. In particular, we focused our attention on two YAP transcriptional targets, namely CYR61 and CTGF, based on evidence from the literature of their key role as EMT inducers (I. Haque et al., *Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis*. Mol Cancer (2011); PMID: 21232118; S. Sonnylal, et al. *Connective tissue growth factor causes EMT-like cell fate changes in vivo and in vitro*. J Cell Sci (2013). PMID: 23525012). Interestingly, we also noted that CYR61 and CTGF are comprised in a list of genes commonly upregulated in the 22-gene YAP transcriptional signature and the EMT signature that we have used throughout our study (see new Supplementary Figure 12a). We found that simultaneous silencing of these two genes results in reversion of the EMT transcriptional in the GSEA analysis of BBN-NUMB-KO cells (see new Supplementary Figure 12b,c) and NUMB-KD human RT4 cells (see new Supplementary Figure 12e,f). These effects were accompanied, in both model systems, by inhibition of the invasive phenotype in the transwell migration assay (see new Suppl. Figure 12d and 12g for mouse BBN tumors and human RT4 cells, respectively). These results point to CYR61 and CTGF as key players of the YAP-dependent activation of EMT, downstream of NUMB dysfunction.

Related to this point, we refer this Reviewer to our response to point 4, where we describe a set of new transcriptomic data based on the use of verteporfin, which clearly indicates that NUMB loss-directed EMT activation is downstream of YAP transcriptional activity.

5)The spacing between the target bands in the immunoblot analysis of Figure 4B appears uneven.
R. Agree. We have amended the old Figure 4b (new Figure 6b in the revised manuscript) to address this Reviewer’s point.

6)Some of the references are incorrectly formatted, e.g:13、14、32、34 et al. Kindly verify and make the necessary adjustments with careful attention.
R. Agree. We believe that some problems might have occurred in the PDF conversion during the submission procedure. However, following the Reviewer’s suggestion, we have carefully checked the formatting of the reference section in the revised manuscript, according to the Nature Communications formatting instructions.

POINT BY POINT REPLY TO THE REVIEWERS' COMMENTS (reproduced verbatim) Reviewer #1

The authors have significantly improved the manuscript and thoroughly addressed my comments, as well as the critics of the two other reviewers. I would like to congratulate them for the amount of work and the high quality of their manuscript, which is elegant both in terms of content and data/figures quality. I believe that the manuscript is worthy of publication in Nature Communications provided that the below minor comments are further addressed/discussed:

R. We would like to thank this Reviewer for the rewarding and appreciative words on our work. We also thank this Reviewer for the insightful criticism and suggestions provided, which helped us to craft an improved version of our manuscript. This Reviewer raised two relevant points that we address below *(the points are reproduced verbatim, maintaining the reviewer's original numbering for convenience)*:

1) "Therefore, the NUMB^{LESS} signature could be used to identify patients with tumors displaying aberrant YAP/EMT activation (line 209)". The therapeutic and translational relevance of the NUMBless signature is also discussed in the discussion part.

If I understood correctly, the authors show that the YAP activation signature has more prognostic/predictive significance than the NUMBless signature (fig. 2f and 1h). What would be the advantage to use a NUMBless signature versus the YAP activation signature then? Is it adding some information or is it "easier" to assess? What about the combination of them? It is difficult to grasp the translational potential specifically related to NUMB in view of the important prognostic/predictive significance of the YAP signaling per se and the fact that YAP is targetable while NUMB is likely not (which does not diminish the mechanistic significance of the study). Thus, I would be careful with such type of statement or come up with a better rationale underlying the translational potential associated with NUMB findings which add value to the known significance of YAP in BCa.

R. Agree. We thank the Reviewer for raising this point as it made us realize that the sentence at line 209 could be misleading, if interpreted as a conclusion of the analyses illustrated in Fig. 1h,i and Fig. 2f,g. Indeed, the overall goal of these analyses was to assess the relevance of the connection between NUMB dysfunction and YAP/EMT activation, identified in our transcriptomic studies in human BCa cell lines, to real-world BCa, in particular to NMIBC patients. Our intent was not to provide a head-to-head comparison of the clinical value of the NUMB^{LESS} *vs.* the YAP signatures, nor to propose the NUMB^{LESS} signature as a surrogate prognostic biomarker for YAP activation. In fact, as the Reviewer correctly noted, the YAP signature has a superior prognostic value compared to the NUMB^{LESS} signature, which is expected given that NUMB dysfunction likely represents only one of the possible mechanisms leading to YAP activation in BCa. Indeed, both canonical and non-canonical YAP activating mechanisms have been described in BCa (as reviewed in X. Cheng *et al.* Front Oncol., 2022; 12: 925278; doi: 10.3389/fonc.2022.925278). In addition, an active YAP status in BCa can also depend on direct amplification of *YAP* itself or loss-of-function/inactivating mutations of its upstream inhibitors, for instance LATS (Y. Wang et al., Cell Reports, 2018: 1304-1317.e5; doi 10.1016/j.celrep.2018.10.001).

Therefore, to address the Reviewer's point, we have now removed this sentence (originally at line 209; see the comment at page 10, line 202, of the revised version). Following the Reviewer's suggestion, we have also better discussed the translational relevance of the NUMB^{LESS} signature, placing emphasis on its potential as a biomarker for the identification of NMIBC patients who, being at high risk of MIBC progression depending on the dysfunction of the NUMB/RHOA/ROCK/YAP axis, might specifically benefit from novel targeted therapies against this circuitry (see the changes

and comments at pages 25-26, lines 596-598 and 612-617 of the revised version). Related to this point, the Reviewer correctly notes that, differently from YAP, NUMB is not *per se* a druggable target. However, the therapeutic actionability of YAP also poses considerable challenges, considering the systemic toxicity and the unpredictable outcomes that might derive from the inhibition of YAP in tissues where it exerts a tumor suppressor rather than a tumor-promoting role (reviewed in Baroja, I. *et al.* Expected and unexpected effects after systemic inhibition of Hippo transcriptional output in cancer. Nat Commun 15, 2700, 2024. https://doi.org/10.1038/s41467-024-46531-1; Moroishi T., *et al.* The Emerging Roles of YAP and TAZ in Cancer. Nat. Rev. Cancer. 2015;15:73–79. doi: 10.1038/nrc3876). In this context, our study highlights an additional site of therapeutic intervention in the YAP signaling pathway, targeting the upstream RHOA/ROCK axis. NUMB dysfunction represents a predictive biomarker of response to targeted anti-RHOA/ROCK (as well as anti-YAP) therapies through the identification of BCa that are addicted to this circuitry for their clinical aggressiveness.

2) Related to non-bladder specific effects of Numb KD in CK5-positive cells: I appreciate the careful examination of the authors in the various organs. Yet it is somehow difficult to understand that targeted Numb silencing in the basal layer of the mouse urothelium is alone sufficient to trigger spontaneous bladder tumorigenesis, while it has 0 effects on other CK5+ tissues (knowing that Numb has been shown to have a role in some of these tissues). The authors discuss results in the breast which can be justified by the differences between males and females. In contrast, it is surprising to observe no effects (after 24 weeks) in male organs such as the prostate (i) which has a full CK5+ basal layer and (ii) in which NUMB has been demonstrated to be potentially important for tumorigenesis. Any explanation?

R. We acknowledge this Reviewer's point. Admittedly, we were equally surprised by the absence of morphological alterations in any other organ but the bladder in our systematic survey of CK5-positive tissues in male mice. We can only propose as a plausible explanation a unique vulnerability of the bladder mucosa to aberrant morphogenesis and neoplastic transformation following NUMB dysfunction, likely depending on the crucial role that NUMB plays in maintaining normal urothelial homeostasis. Put differently, there might exist intrinsic differences in the susceptibility of different epithelia to undergo malignant transformation following NUMB loss. These differences could be linked to the context-specific regulation (i.e., hyperactivation or downregulation) of different pathways epistatically controlled by NUMB, as well as the complex reciprocal synergistic or antagonistic interactions between these pathways. In support of this argument, we note, for instance, that the NOTCH pathway, identified in several studies as a driver oncogene in prostate tumorigenesis and proposed as a potential therapeutic target (see the review V.A. Belle *et al. NUMB inhibition of NOTCH signalling as a therapeutic target in prostate cancer*. Nat Rev Urol. 2014 Sep; 11(9): 499–507. doi: 10.1038/nrurol.2014.195, and references therein), is downregulated, rather than hyperactivated, in our NUMB-deficient human and mouse BCa models (see also our response to point #4 of this Reviewer in our point-to-point response in the previous round of revision). In contrast to NOTCH, while the YAP pathway has a well-defined oncogenic role in bladder tumorigenesis, representing a hallmark of biologically and clinically aggressive disease (our study and references herein), it appears to play a tumor suppressor role in prostate tumorigenesis, where YAP can inhibit prostate cancer growth by antagonizing TEAD-mediated androgen receptor signaling (X. Li *et al.* YAP antagonizes TEAD-mediated AR signaling and prostate cancer growth. EMBO J, 2023, 42: e112184; doi.org/10.15252/embj.2022112184).

An additional factor potentially contributing to the intrinsic difference in the sensitivity of the bladder and the prostate to NUMB-driven neoplastic transformation is the crucial role that chronic inflammation plays in prostate tumorigenesis. This common urological condition affects half of all men in their lifetimes, and is a major risk factor for the development of both benign and malignant prostate tumors (reviewed in A.M. De Marzo *et al.*, Inflammation in prostate carcinogenesis. Nat Rev Cancer. 2007 Apr; 7(4): 256–269. doi: 10.1038/nrc2090). For instance, in mouse models, a bacterial-

induced chronic prostate inflammation drives a basal -to-luminal differentiation process that accelerates tumorigenesis, supporting a unique role for inflammation in cancer initiation (O-J Kwon *et al*. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin. *Proc Natl Acad Sci USA* 111, E592–E600, 2014. doi: 10.1073/pnas.1318157111). It would be interesting to investigate whether, in a model of chronic prostate inflammation, NUMB dysfunction favors the disruption of prostate homeostasis and induce early onset and progression of hyperplastic or even neoplastic alterations. In addition, it would be interesting to determine the reciprocal contribution of NOTCH and YAP signaling in this process.

3) Please, double-check the few typos and conjugation mistakes throughout the text (e.g. Line 433 "Supporting this hypothesis, treatment with Y-27632 reversed the aberrant morphology of NUMB-KO MBOs and markedly decreased YAP transcriptional activity (Supplementary Fig. 9i), while have no significant effects on WT MBOs...)
R. Agree. We thank the Reviewer for making us note these mistakes. We have amended the text as appropriate.

Reviewer #2
In the previous version, the study by Tucci et al had provided important new insights into the role of NUMB in bladder cancer. The revised manuscript, in this reviewer's opinion, has been greatly improved by addressing the comments of all three reviewers. This is an outstanding revision and outstanding manuscript. I have no further suggestions.

R. We are most grateful to this Reviewer for the appreciative words on our study and for the suggestions provided, which helped us to produce an improved version of our manuscript, in particular in relation to is potential translational relevance.

Reviewer #3
I and co-reviewer agreed that authors have successfully addressed the reviewers' comments.
We recommend the manuscript to be accepted for publication.

Reviewer #4 (ECR co-reviewer)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

R. We thank these two Reviewers for their insightful suggestions and detailed indications provided in the previous round of revision, which helped us to address important mechanistic aspects in our revised study.