nature portfolio

Peer Review File

Ligand-dependent Wnt signaling promotes gastric cancer metastasis through hyaluronan expression in microenvironment

Corresponding Author: Professor Masanobu Oshima

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors identified a new role for WNT ligands in promoting liver metastasis of gastric cancer, utilizing genetically engineered organoids, transplantation models, and spatial transcriptomics (Visium). It was proposed that canonical Wnt ligands (specifically Wnt1) secreted by tumor cells upregulate hyaluronan synthase, Has2, in cancer-associated fibroblasts (CAFs) derived from hepatic stellate cells, thereby promoting liver metastasis. This is an interesting study that unveils an unexpected role of canonical WNT ligands in establishing a tumor-favoring metastatic niche. However, several points warrant further attention through new experimental results, discussion, and tone adjustments.

Major comments

1. Orthotopic vs. intrasplenic transplantation. With the exception of gastric tumor analysis (Figure 2), the authors performed intrasplenic injection of organoids instead of orthotopic transplantation. Consequently, this study does not provide direct evidence on whether orthotopic injection of KTP or WKTP organoids into the stomach would result in metastasis to other organs. It is necessary to assess whether orthotopically injected organoids/cells give rise to distant metastases, particularly to the liver, followed by repeating key experiments shown in Figures 3-7 using orthotopic models.

2. Immunocompetent vs. immunocompromised mice: The authors utilized NSG and SHO mice for transplantations. Given that KTP and WKTP organoids were derived from C57BL/6 mice, syngeneic transplantation in C57BL/6 mice could offer a more pathophysiologically relevant immune environment. Could the authors clarify their rationale for choosing an immunodeficient background instead? Considering the aforementioned point (#1), performing orthotopic transplantation of organoids (or organoid-derived cells) in C57BL/6 mice is necessary. Alternatively, investigating liver metastasis in the genetically engineered mouse models (GEMMs) (Figure 1) could also be considered, as the authors described in the Introduction (lines 92-96; notably, this GEMM was not used to study metastasis in this manuscript).

3. Mechanisms: How does WNT/β-catenin signaling convert HSCs into CAFs and upregulate Has1/2? More discussion is needed.

4. HAS2 blockade in tumor cells (performed in this manuscript) vs. CAFs (not presented): Instead of Hyal expression in WKTP, HAS2 blockade in CAFs would be ideal. Thus, tone adjustment or further discussion is recommended.

5. Pathological relevance: The study focuses on liver metastasis, but GC is known to metastasize to various organs, including the peritoneum, lymph nodes, and lungs. The authors must clarify whether liver metastasis is the most clinically relevant metastatic site for GCs expressing canonical Wnt ligands.

Minor comments

1. The Rationale for selecting Wnt1 (over other ligands, including Wnt3a and Wnt5a). This study mentioned the role of ligand-dependent Wnt signaling in GC, and Wnt1 was specifically chosen for transgenic expression in the mouse model. However, it remains unclear why Wnt1 was selected over Wnt3a or Wnt5a. The authors need to provide literature, dataset,

or experimental results-based justification for prioritizing Wnt1. Additionally, is there any transcriptomic or patient-derived data supporting WNT1 as the dominant Wnt ligand in liver-metastatic gastric cancer or a CIN-subtype? It should be noted that Wnt5a is a non-canonical WNT ligand. Similarly, in line 278, it is necessary to separate canonical vs. non-canonical WNT ligands since this study suggests the crucial role of canonical WNT ligands in liver metastasis.

2. Figures 6 and 7: What is the primary source of TGF-beta to activate CAFs and upregulate HAS in conjunction with canonical WNT ligands?

Reviewer #2

(Remarks to the Author)
The manuscript demonstrates that Wnt ligand-dependent signaling promotes metastasis of chromosomal instability (CIN) subtype gastric cancer to liver through increasing hyaluronan expression in the metastatic tumor microenvironment. This study used multiple mouse models with mutations driven by Claudin18-IRES-CreERT2, inducing Tgfbr2flox/flox, Trp53LSL·R270H, KrasLSL·G12D, and K19-Wnt1. The organoids derived from these mouse models highlighted that Wnt1 signaling activation is required for efficient liver metastasis in stromal cells.
The current manuscript could be the first study detailing the potential mechanism of how stromal Wnt1 promotes CIN gastric cancer metastasis via Has2. The experiments are very nicely illustrated, and the data are almost all of high quality. The paper is also clearly written. However, there are several concerns that should be addressed:

1. The study used a tamoxifen (Tam) concentration of 4mg/mouse to induce CreERT2 at 6 weeks of age. Tam at this concentration can cause parietal cell loss and induce chief cells to go through spasmolytic polypeptide-expressing metaplasia (SPEM). Authors should discuss the possible stomach epithelial injury resulting from use of Tam at this concentration and its potential impacts on their gastric cancer genetic engineered mouse model and cite the relevant papers: PMID: 22001866, 27246044, 31233898. It is possible the metaplasia caused by Tam helps give a headstart to the lesions/tumors.

2. How did the authors activate CreERT2 in vitro? Tamoxifen is usually not miscible in tissue culture media, so most people use 4-hydroxy tamoxifen instead of tamoxifen.

3. In Figure 1A, authors should clarify the time needed for Tam to induce KTP mutations in organoids. Also, “KP” is an error, as it is the same as “KT”.

4. For the invasive lesions in Fig. 1, how confident are the authors that the glandular forms found, especially solitary ones are actually invasive? Because when there is marked hyperplasia of mouse stomach, the tissue can be distorted such that glands can twist around and be cut in cross-section, appearing pseudo-invasive. Although the image is small, it does look as though the single gland invasion in 1G is invasive, because there is distortion of surrounding stromal cells, and there seems to be an actual tumor (mass) formed, but this call can be tricky in many cases.

5. There seems to be a loss of Muc5AC-positivity in the pit zone of Wnt-expressing tumor mice. What makes up the pit in these mice? Is there a reason why overexpression of Wnt would block foveolar cell differentiation?

6. The mouse genetics are a bit unclear. Is the Trp53R270H allele kept as a heterozygote with the other, endogenous Trp53 allele wild-type? Presumably, the Kras gain of function is an LSL?

7. Figure 2C is missing the statistical analysis.

8. Figure 3 and Figure 4: for the KTP/WKTP/Apc-KTP/Wnt-KTP spleen injection experiment, besides in the liver, is there any other organ metastasis? Especially in the WKTP group, the liver metastasis phenotype is very strong as shown in Figure 4F.
Figure 3A: typo, “speen” should be “spleen”.

9. Figure 3F: needs to show the representative H&E images of tumor area/field. Moreover, how are we to interpret reproducibility/statistics here? One control mouse is lower than all 3 in GNF. Are the datapoints means from many tumor areas/fields in a single mouse? There is no stat analysis here either.

10. Figure 3H: authors used GFP to trace tumor cells. But where is the GFP coming from? Authors did not mention the use of GFP in the mouse strains. Authors should clarify all the detailed genotypes of the mouse strains used in current manuscript (W, K, KT, KP, KTGP, WKT, WKP, WKTP).

11. Figure 4C needs to label the protein size (kDa). Why not western blot for APC and Wnt1?

12. Figure 4F/Supplementary Figure 5B experiment need the β-catenin IHC staining results. Are there more nuclear β-catenin in stromal cells in the Wnt-KTP group compared to KTP or Apc-KTP groups?

13. The labels among the groups in Figure 4F and Figure 4G are confusing. There is one group of KTP mice in Figure 4F, but with two columns for KTP mice (#1,2) shown in Figure 4G; and one group of WKTP mice in Figure 4F but with three columns shown in Figure 4G (#1,2,3). In Figure 4F, the KTP and WKTP image section is missing a scale bar.

14. The authors should perform a transwell assay to test the cell invasion ability among KTP, WKTP, Apc-KTP, and Wnt-KTP groups.

15. Supplementary Figure 5A is missing Wnt-KTP cell data. How did authors reach the conclusion that “the proliferation rates of Apc-KTP and Wnt-KTP cells were unchanged compared to parental KTP cells”?

16. Figure 5B, 5D and Supplementary Figure 6 are missing a scale bar.

17. “Hepatocyte marker Alb was significantly increased in cluster 3 and 6, while epithelial markers, Ceacam1 and Muc1, were increased…” Technically, liver is epithelium also, so this should be clarified, because it is not clear which epithelial markers were used or why the ones shown are the representative epithelial cell markers.

18. The authors should show representative IHC image results (translational level) to validate the spatial transcriptomic data (transcriptional level), for CTNNB1, WNT3A, TGFB1, SAMD3, and SMAD4.

19. In Figure 7A, Has2 is not limited to cluster 5, which raises the question of whether the authors have performed the filtering before analyzing the spatial transcriptomic data by using Loupe Browser? The authors did not mention how they processed the spatial transcriptomic data. This should be detailed.

20. The authors mention HA interacting with CD44 on stem cells and hypothesize the same sort of interaction in gastric cancer, but there is a paper that does show that metaplasia in stomach expands CD44+ progenitor cells that as HA expands, so this paper should be cited and potentially discussed: PMID:23589310. There are other papers detailing CD44 role in metaplasia and stemness in stomach (eg, PMID: 31071489), so the CD44/xCT/HA pathway may be important in both metaplasia and metastasis.

21. A visualization of cell-cell communication, using differentially expressed genes (DEGs), would greatly improve analysis, as one would expect important cell-cell communication between cluster 5 and the rest of the clusters. For example, as the authors state, CD44 in tumor cells could be communicating with HA in stroma.

Reviewer #3

(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.

Reviewer #4

(Remarks to the Author)
Furutani et al engineered a mouse model of gastric cancer expressing mutated
Kras, Tgfbr2, and Trp53 (KTP mice) specifically in the gastric mucosa. These mice displayed gastric metaplasia with parietal cell loss. In a second GEMM the same driver mutations were expressed together with Wnt1 (WKTP mice) and these mice developed dysplastic tumors. To investigate the role of ligand-dependent Wnt signaling in gastric cancer, they established organoids from the gastric epithelia of these mice, transplanted them into the spleens of NSG mice and observed that while both KTP and WKTP organoids formed local splenic tumors, only WKTP organoids could form liver metastases. Using a combination of approaches, including spatial transcriptomic, they found that in mice implanted with WKPT organoids, liver metastases were associated with a significant stromal response and identified hyaluronan synthase (Has2) as a potential mediator of this response. Furthermore, they found a significant hyaluronan deposition in liver metastases of both mouse and human gastric cancers. The authors concluded that paracrine Wnt signaling activation in hepatic stellate cells in conjunction with TGFβ results in increased Has2 expression and this promotes metastatic tumor development through hyaluronan deposition and increased ECM production. This study follows an earlier one by the same group where the role of these driver mutations in formation of intestinal tumors was analyzed (Kok et al, 2021).

A similar approach to generating gastric cancer GEMMs was published by Seidlitz T. et al in 2019 so the strategy used is not entirely novel. However, the present study adds novelty, by identifying a major role for Wnt ligands in shaping the liver microenvironment and thereby, enabling gastric cancer liver metastases.

Technically, the experiments were well executed, and the manuscript is (with a few exceptions) well written. However, there are several issues that need to be addressed
General comment:
In Seidlitz et al, a similarly constructed GEMM based on driver mutations KrasG12D/+;Tp53R172H/+;Smad4fl/f in the gastric epithelium resulted in gastric tumors that were metastatic to liver and lung even in the (apparent) absence of Wnt production. These findings are at variance with the results obtained in the present study with the KTP mice and the authors should discuss potential differences that could explain the different phenotypes.
Specific points
Figure 2 (E-H). This model of orthotopic implantation best mimics the clinical pathology. Did the authors analyze progression to metastatic disease in these mice? Do WKTP tumors spontaneously metastasize to the liver in this setting? If not, a

discussion is warranted.
Figure 3F - Were the differences between the different group statistically significant? If not, this should be stated. There appears to be a high variability in the data obtained for the control group and analysis of additional mice may be necessary to support the conclusion that these inhibitors had an effect.

Figure 3 (H&I) – Among the earliest events in liver metastases formation is the recruitment of innate immune cells, among them neutrophils and monocytes which are still functional in NSG mice. Wnt1 is known to affect immune cell function including macrophage polarization (e.g. Staal et al, 2008, Haseeb M et al 2109). Given the results shown this figure, the authors should analyze or at least comment on the potential role of myeloid-derived cells in promoting establishment of metastases of WKPT cells.

Fig 4C- The ratio of activated:total beta-catenin appears to be higher in KTP than in WKTP cells. This is surprising. Is there no autocrine effect of Wnt1 in the WKTP cells? Does Wnt1 only act in a paracrine fashion and specifically only in the liver? Did the authors quantify Wnt1 production levels in the three models (KPT, WKPT and Wnt-KPT)? These data are important for interpretation of the results.

Figure 4F- How do the authors explain the striking difference in the extent of liver metastatic disease in WKPT and Wnt-KPT implanted mice? Were Wnt1 production levels in these cells compared?

Figure 5- To determine whether the extensive stromal response observed in these livers is a cause or consequence of metastatic expansion, livers should also be analyzed at earlier time points. It is also of interest to know whether the spatial transcriptomic analysis identified any myeloid/innate immune cell clusters and whether liver sections of KPT -implanted mice were also analyzed for comparison.
Discussion and data interpretation
The authors propose that the ability of KPT and WKPT organoid to form tumors and grow equally well in the orthotopic (and subcutaneous) site is due to local Wnt1 production by stromal cells. This is speculative and should be experimentally tested, for example by using the PORCN Wnt1 inhibitor C59. This also raises the question why the authors switched from an orthotopic to an intra-splenic model to evaluate liver metastasis. If WKPT cells do not metastasize following intra-mucosal implantation (or metastasis is a very late event) this should be stated and discussed.

The functional significance of hyaluronan in metastatic expansion in the liver in this model has not been defined. Is it utilized by WKPT cells to attach via CD44?
Whether the fibrotic response is a cause or consequence of tumor expansion in the liver should be resolved experimentally by analyzing the response on day 7 not at late stages of metastasis as demonstrated in Fig 5.
Language editing is required. For example see line 329.

Reviewer #5

(Remarks to the Author)
Furutani and colleagues investigated the role of ligand-dependent Wnt signaling in gastric cancer development and metastasis by creating genetically modified mice (KTP and WKTP) with or without Wnt1 expression. They found that WKTP mice developed dysplastic tumors and liver metastases, while KTP mice only showed gastric metaplasia, highlighting Wnt signaling's role in both tumor initiation and metastasis. Using organoid transplantation and spatial transcriptomics, they showed that stromal Wnt signaling, together with TGFβ, activates hepatic stellate cells into cancer-associated fibroblasts that express Has2, promoting hyaluronan accumulation and metastasis. Importantly, inhibiting Has2 or Wnt signaling reduced liver metastases, suggesting a potential therapeutic approach for gastric cancer.

General Remark: While the study is interesting, there is very little evidence that the findings could be translated to human (very little has been done to validate the findings in human). While the study uses an experimentally sophisticated system (many transgenic animals) there is an important number of issues that needs to be carefully addressed. When talking about Wnt-signaling and paracrine communication between cancer cells and CAF it is important to note that specific Wnt ligands will be secreted by specific cells while specific receptors will be expressed on certain cells. This is where the study in reviewers opinion is limited and to certain extent far from realistic situation encountered in human.

Major Remark 1: In human gastric cancer HAS2 is not expressed on stellate cell derived CAF. Namely, there are generally 2 types of CAF (in the liver) depending on their origin, stellate cell derived (RGS5+) or resident fibroblast derived (PDGFRA+). Hence the model that is used in vitro (employing immortalised HSC) is unfortunately irrelevant. This goes further, HYAL2 is not expressed by cancer cells, but by endothelial cells while Wnt5A is expressed by cancer cells.

I am attaching in support some meta-analysis that I have conducted based on previously published data (DOI:10.1002/ctm2.730)

In conclusion, the authors need to be very careful about which ligand is expressed on which cell (lay out their model based on human data, either single cell if clear enough or IHC). Then according to this design their experimental strategy. Concretely, which cells express WNT1 in human gastric cancer and what is the target receptor(s), where are these receptors situated in humans?

Major Remark 2: The part of the data that is related to CAF needs thorough revision. The authors state "It has been shown

that CAFs in the liver metastasis are originated from activated hepatic stellate cells (HSCs)", which is simply wrong in many aspects (the cited literatures is not relevant, please see doi: 10.1038/s41586-019-1631-3.). Again, CAFs in the liver metastasis are derived from at least 3 different precursor cells - and in contrast to what the authors are claiming, those CAF that reorganise the ECM are not stellate cell derived. I further refer to the attached meta-analysis where on primary gastric cancer samples from human we can clearly see at least 2 groups of CAF (stellate cells do exist also in stomach and many other organs next to liver) to begin with.

Major Remark 3: Provided that the authors sort out the cellular identities according to the remarks made above, what is the link between Wnt-signalling and HAS2 expression? The authors switch without any explanation from Wnt1 (which is their initial model) to Wnt3. What is the reason for this? What is the downstream signalling and how Wnt1 (and not 3) could cause the HAS2 over-expression?

Major Remark 4: Hyaluronidase inhibitors have been shown to inhibit cancer metastasis (https://www.nature.com/articles/s41598-024-64924-6). The authors here show the contrary. It seems that high molecular weight hyaluronic acid (HMW-HA) is tumor protective, while low molecular weight hyaluronic acid (LMW HA) is tumor promotive. Authors need to clarify better which type of HA they are dealing with and relate this to what is observed in human samples.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have done a very scholarly job of addressing reviewer concerns. Here are some minor comments:

1. It would be better to mention in the manuscript why authors chose to assess Wnt pathway activity by detecting the active form of β-catenin via immunoblotting and by performing the TOPFLASH reporter assay.

2. In manuscript lines 213–214, the authors stated: “Importantly, all three independently established Apc-KTP organoid cell lines did not develop liver metastasis, and only rare small clusters of tumor cells were detected.” However, in the current Figure 4f (Apc-KTP#2), liver metastases are visible at multiple locations: in the first liver (5 o’clock and 11 o’clock), the second liver (1 o’clock), and the third liver (3 o’clock and 11 o’clock), with tumors present at the liver edges. In addition, Supplementary Figure 6C clearly shows liver metastases in the Apc-KTP group. Therefore, the statement in lines 213–214 is inconsistent with the presented data and should be revised.

3. The reason for using a splenic transplant (vs true orthotopic) model as well as not using C57/Bl6 mice as hosts might be mentioned in the final text and not just in the response to reviewers, as some readers may also be curious.

Reviewer #3

(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.

Reviewer #4

(Remarks to the Author)
The authors satisfactorily addressed most of my concerns. A better characterization of the polarization state of recruited macrophages in their GEMMs could further improve the understanding of the role of immune cells in liver metastasis in these models.

Reviewer #5

(Remarks to the Author)
Furutani et al have provided the revised manuscript. Overall reviewers comments regarding the consideration of relevant literature, CAF-related language have been corrected. However, the key problems have not been experimentally addressed (except the new CAF in vitro experiments in the Fig. 6B and 7B). Unfortunately, two key points remain unanswered and that is:

1) relevance to human: only very limited data, with reduced statistical significance, have been provided from human samples to indicate the some of the observations might hold true in human samples.

2) take more care about the cell of origin. It is not OK to use cancer cells to express stromal genes even if these are supposed to be secreted and exert their activity outside the cell. One does not know if these proteins may have functions in the cytoplasm. Hence, if HYAL1 is expressed in endothelial cells, then a model must account for that and expressing HYAL1 in cancer cells will not be considered as relevant.

Next to this, there are several points that need to be addressed to clarify if the data from the manuscript could be reproduced in the future. In particular the authors should:

1) make sure that reviewers now and other scientist later can access the OMICS data. The link that has been provided does not work.

2) the WKTP gastric cancer model, as well as all other models that are not publicly available yet used in the present manuscript, need to be outlined in the "Material Availability" section, where the conditions and accessibility for sharing these models should be clearly stated.

Reviewer #6

(Remarks to the Author)
Furutani and colleagues present a compelling study identifying a novel role for Wnt1-dependent signaling in conjunction with hyaluronan-expressing cancer-associated fibroblasts as a key mediator of gastric cancer liver metastasis. Using diverse methodologies, they demonstrate that cell-intrinsic Wnt signaling alone is insufficient for metastasis induction; instead, synergistic Wnt and TGFβ signaling induces hyaluronan in stromal cells. The study is strengthened by robust genetic control populations and inhibition models. The authors adequately addressed previous reviewer concerns, with manuscript revisions enhancing the overall quality, and I support its publication in Nature Communications.

Minor concerns are outlined below.

1) Lines 113–114 claim all mutant mice showed significantly increased thickness, but the statistical comparison of WT vs. K is missing in Figure 1c.
2) Supplementary Figure 8b shows a correlation between HAS2 and WNT6, but WNT6 is not mentioned in the text.
3) Figures 6 and 7 label “Afamin-Wnt3,” while the text uses “Afamin-Wnt3a.” Relabel figures as “Afamin-Wnt3a” for consistency.
4) The response to Reviewer #1’s concern about using immunodeficient vs. immunocompetent mice is well-articulated. The referenced study (Sakai et al., Cancer Res, 2018) should be cited in the paper, and including parts of this explanation in the discussion would strengthen the experimental design and conclusions.

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)
I note the text modifications the authors have made. The reviewer believes that experiments would have been more appropriate. The reviewer has no further comments.

Response to the reviewer comments

Our point-by-point responses to the comments made by the Reviewers are included below. The comments are quoted "verbatim", followed by our responses.

Reviewer #1 (Remarks to the Author)

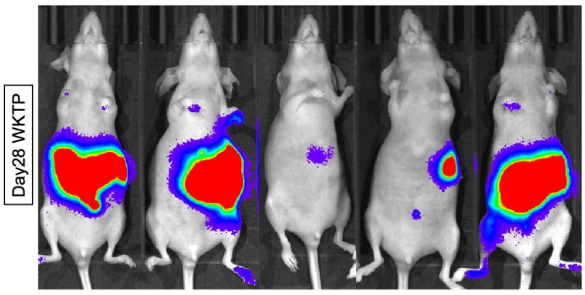
The authors identified a new role for WNT ligands in promoting liver metastasis of gastric cancer, utilizing genetically engineered organoids, transplantation models, and spatial transcriptomics (Visium). It was proposed that canonical Wnt ligands (specifically Wnt1) secreted by tumor cells upregulate hyaluronan synthase, Has2, in cancer-associated fibroblasts (CAFs) derived from hepatic stellate cells, thereby promoting liver metastasis. This is an interesting study that unveils an unexpected role of canonical WNT ligands in establishing a tumor-favoring metastatic niche. However, several points warrant further attention through new experimental results, discussion, and tone adjustments.

Response➔We have responded to all comments by Reviewer #1 as follows.

1. Orthotopic vs. intrasplenic transplantation. With the exception of gastric tumor analysis (Figure 2), the authors performed intrasplenic injection of organoids instead of orthotopic transplantation. Consequently, this study does not provide direct evidence on whether orthotopic injection of KTP or WKTP organoids into the stomach would result in metastasis to other organs. It is necessary to assess whether orthotopically injected organoids/cells give rise to distant metastases, particularly to the liver, followed by repeating key experiments shown in Figures 3-7 using orthotopic models.

Response➔We thank the reviewer for this important comment. In our orthotopic transplantation experiments, we monitored gastric tumor development using in vivo imaging up to day 21, as shown in Figure 2g. At this time point, no clear evidence of metastasis was observed. However, by day 28, some mice began to exhibit signs of peritoneal dissemination, as demonstrated in the imaging data provided below. Although histological analysis at this stage did not reveal liver metastases, the mice showed signs of clinical deterioration, limiting our ability to extend observation further.

To establish a more quantitative and reproducible model for assessing liver metastasis, we therefore employed the intrasplenic injection approach. As the reviewer rightly noted, this model bypasses early steps of the metastatic cascade, particularly invasion and



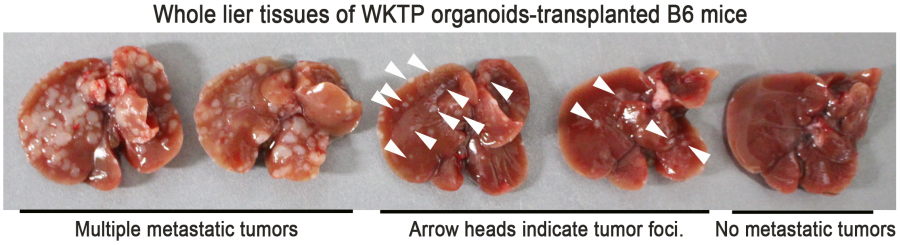
intravasation from the primary site. Nonetheless, it enables robust evaluation of the role of Wnt ligands in supporting metastatic outgrowth in the liver microenvironment.

To clarify this point in the manuscript, we have revised the sentence in the Results section (**page 7**) as follows: “To investigate the role of ligand-dependent Wnt signaling in liver metastasis by disseminated tumor cells, we transplanted KTP and WKTP organoid cells into the spleen of mice and analyzed liver tissues.” We hope this explanation and the complementary use of both models address the reviewer’s concerns and support the rationale behind our experimental design.

2. Immunocompetent vs. immunocompromised mice: The authors utilized NSG and SHO mice for transplantations. Given that KTP and WKTP organoids were derived from C57BL/6 mice, syngeneic transplantation in C57BL/6 mice could offer a more pathophysiologically relevant immune environment. Could the authors clarify their rationale for choosing an immunodeficient background instead? Considering the aforementioned point (#1), performing orthotopic transplantation of organoids (or organoid-derived cells) in C57BL/6 mice is necessary. Alternatively, investigating liver metastasis in the genetically engineered mouse models (GEMMs) (Figure 1) could also be considered, as the authors described in the Introduction (lines 92-96; notably, this GEMM was not used to study metastasis in this manuscript).

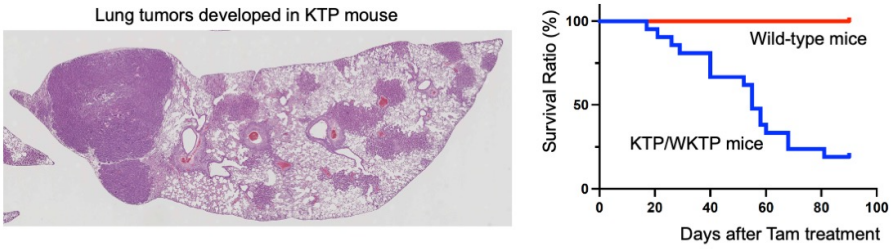
Response➔In our previous study (Sakai et al., Cancer Res, 2018), we established AKTP organoids derived from intestinal tumors in C57BL/6 (B6) mice, harboring mutations in *Apc*, *Kras*, *Tgfbr2*, and *Trp53*. In that study, we found that intrasplenic injection of AKTP cells into syngeneic B6 mice resulted in liver metastases in approximately 40% of the animals, whereas injection into immunodeficient NSG mice led to liver metastasis in nearly 100% of recipients. This discrepancy is likely due to the NK cell-mediated surveillance in B6 mice, although it remains to be elucidated. Based on this finding, we selected NSG mice for the current metastasis experiments to ensure high reproducibility and efficiency in detecting metastatic phenotypes, particularly in comparing the metastatic potential of KTP and WKTP organoids.

In response to the reviewer’s suggestion, we performed intrasplenic transplantation of WKTP organoid cells into immunocompetent B6 mice. As shown in



the representative photograph below, multiple liver metastasis developed in 2 out of 5 mice, and low-multiplicity liver tumors were observed in additional 2 mice. In contrast, consistent with our previous observation, WKTP cells give rise to multiple liver metastatic in 100% of NSG (Fig. 3f, 4f, 4g). These results support the enhanced sensitivity of the NSG mice for studying Wnt ligand-associated metastasis mechanism.

Regarding the potential use of GEMMs to study spontaneous metastasis, we conducted long-term observation (up to 90 days) of both KTP and WKTP mice. While both genotypes eventually developed lung tumors and became moribund likely due to CreER expression in the lung, no spontaneous liver metastases were observed during this period. Histological images of lung tumors and corresponding survival curves are provided below. Thus, to directly assess the metastatic potential of KTP and WKTP cells, transplantation-based models were necessary. We hope this explanation clarifies the rationale behind our use of NSG mice for the analysis and highlights the technical challenges associated with spontaneous metastasis in GEMMs.



3. Mechanisms: How does WNT/β-catenin signaling convert HSCs into CAFs and upregulate Has1/2? More discussion is needed.

Response➔It has been reported that TGFβ signaling activates fibroblasts and induces ECM components in part by downregulating the Wnt antagonist DKK1, thereby enhancing Wnt/β-catenin signaling and contributing to TGFβ-induced CAF activation (Akhmetshina et al., Nat Commun, 2012, *Ref. 33*). Moreover, Wnt/β-catenin signaling has been shown to directly regulate Has2 expression through TCF7L2 binding to the Has2 gene promoter (Sun et al., Biomolecules, 2022, *Ref. 34*). These findings support a model in which Wnt and TGFβ signaling synergistically promote the conversion of HSCs into CAFs and induction of *Has2* expression. We have added these mechanistic insights into the Discussion section, along with newly added references (**page 15**).

4. HAS2 blockade in tumor cells (performed in this manuscript) vs. CAFs (not presented): Instead of Hyal expression in WKTP, HAS2 blockade in CAFs would be

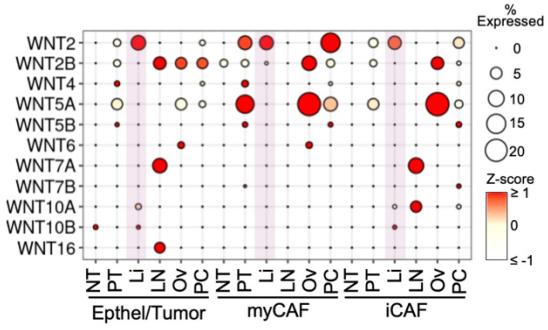
ideal. Thus, tone adjustment or further discussion is recommended.

Response➔As the reviewer pointed out, assessing the effect of Has2 inhibition specifically in CAFs would indeed provide more direct evidence of its role in the tumor microenvironment. However, generating a conditional *Has2* knockout model in stromal fibroblasts, such as HSCs, would require a substantial amount of time. In line with the reviewer's recommendation, we have accordingly moderated the tone of our conclusions (**page 15**) and added a discussion in the revised manuscript, highlighting the need for future studies to investigate the functional relevance of Has2 in CAFs using more targeted approaches (**page 16**).

5. Pathological relevance: The study focuses on liver metastasis, but GC is known to metastasize to various organs, including the peritoneum, lymph nodes, and lungs. The authors must clarify whether liver metastasis is the most clinically relevant metastatic site for GCs expressing canonical Wnt ligands.

Response➔We thank the reviewer for pointing out the organotropism of gastric cancer (GC) metastasis. It is well established that GC metastasizes to various organs, including the liver, peritoneum, lymph nodes, and lungs. Among these, liver and peritoneal metastases are the most frequent.

To address the reviewer's comment, we analyzed publicly available single-cell RNA-seq datasets of GC (GSE163558, GSE239676, and GSE246662) and examined the expression of Wnt ligand family genes in primary tumor cells (PT), metastatic tumor cells from liver (Li), lymph nodes (LN), ovary (Ov), and peritoneum (PC), and cancer-associated fibroblasts (CAFs). As shown in the figure below, WNT2–a canonical Wnt ligand–is highly expressed in the liver metastasis, while WNT2B expression is increased in LN, Ov, and PC metastasis. Thus, no clear correlation can be established between canonical Wnt ligand expression and liver metastasis by this analysis.



According to TCGA classifications, GC exhibits significant genomic heterogeneity and is categorized into four molecular subtypes. Our current mouse and organoid models are based on the CIN subtype, which is more frequently associated with liver metastasis compared to the diffuse-type, which often harbors *CDH1* mutations and prone to peritoneal dissemination (Lee JE, et al., Br J Cancer, 129, 672-682, 2023).

Furthermore, a previous study using GC-derived organoids (Nanki et al, Cell,

2018, *Ref.10*) did not identify a significant association between Wnt ligand expression and GC histological subtype. Thus, whether Wnt ligand-expressing GC cells preferentially metastasize to the liver remains unsolved and is likely influenced by specific driver gene alterations.

To further investigate this, we are currently generating *Wnt1*-expressing, CDH1-deficient GC organoids to assess the role of Wnt ligand signaling in peritoneal dissemination. Given these considerations, we believe it would be inappropriate to generalize that GC cells expressing Wnt ligands preferentially metastasize to the liver in the present study. We hope the reviewer understands our rationale.

Minor comments

1. The Rationale for selecting Wnt1 (over other ligands, including Wnt3a and Wnt5a). This study mentioned the role of ligand-dependent Wnt signaling in GC, and Wnt1 was specifically chosen for transgenic expression in the mouse model. However, it remains unclear why Wnt1 was selected over Wnt3a or Wnt5a. The authors need to provide literature, dataset, or experimental results-based justification for prioritizing Wnt1. Additionally, is there any transcriptomic or patient-derived data supporting WNT1 as the dominant Wnt ligand in liver-metastatic gastric cancer or a CIN-subtype? It should be noted that Wnt5a is a non-canonical WNT ligand. Similarly, in line 278, it is necessary to separate canonical vs. non-canonical WNT ligands since this study suggests the crucial role of canonical WNT ligands in liver metastasis.

Response➔As indicated in Supplementary Fig.1, all Wnt ligand family genes-except Wnt8A-are upregulated in more than 10% of gastric cancers. The upregulation frequencies are comparable among several canonical Wnt ligands, including Wnt1, Wnt2, Wnt3, and Wnt3a. Among these, we selected Wnt1 for this study based on several supporting findings. Previous studies have reported a strong correlation between Wnt1 expression and advanced tumor grade in gastric cancer, and Wnt1-mediated signaling has been shown to promote gastric tumor growth and metastasis (Li et al, Lab Invest, 2023, *Ref. 12*; Mao et al, Cell Death and Disease, 2014, *Ref. 18*). In response to the reviewer's comment, we have included this rationale in the Results section, along with newly added reference (**page 5**).

Regarding the distinction between canonical and non-canonical Wnt ligands as raised by the reviewer, we have revised the explanation for Supplementary Fig. 8b to clearly distinguish the canonical from non-canonical Wnt pathway, highlighting the specific focus of this study on canonical Wnt signaling in the context of liver metastasis of gastric cancer (**page 12**).

2. Figures 6 and 7: What is the primary source of TGF-beta to activate CAFs and upregulate HAS in conjunction with canonical WNT ligands?

Response➔ We confirmed the expression of TGF-β1 in both WKTP tumor cells and CAFs by Western blotting. Moreover, analysis of the VISIUM spatial transcriptomic data revealed that TGF-β1 expression is highly enriched in clusters 1 and 2 (tumor cells), as well as clusters 4 and 5 (stromal fibroblasts). Therefore, we consider WKTP tumor cells to be a primary source of TGF-β ligands in the metastatic lesions. In addition, activated stromal cells subsequently express TGF-β, contributing to sustained activation of TGFβ signaling pathway. These findings have been included **Supplementary Figure 7c** and described in the Results section (**page 11**).

Reviewer #2 (Remarks to the Author)

The manuscript demonstrates that Wnt ligand-dependent signaling promotes metastasis of chromosomal instability (CIN) subtype gastric cancer to liver through increasing hyaluronan expression in the metastatic tumor microenvironment. This study used multiple mouse models with mutations driven by Claudin18-IRES-CreERT2, inducing Tgfbr2flox/flox, Trp53LSL·R270H, KrasLSL·G12D, and K19-Wnt1. The organoids derived from these mouse models highlighted that Wnt1 signaling activation is required for efficient liver metastasis in stromal cells.

The current manuscript could be the first study detailing the potential mechanism of how stromal Wnt1 promotes CIN gastric cancer metastasis via Has2. The experiments are very nicely illustrated, and the data are almost all of high quality. The paper is also clearly written. However, there are several concerns that should be addressed:

Response➔We would like to thank the Reviewer #2 for constructive comments on our manuscript. We have responded to all comments as follows.

1. The study used a tamoxifen (Tam) concentration of 4mg/mouse to induce CreERT2 at 6 weeks of age. Tam at this concentration can cause parietal cell loss and induce chief cells to go through spasmolytic polypeptide-expressing metaplasia (SPEM). Authors should discuss the possible stomach epithelial injury resulting from use of Tam at this concentration and its potential impacts on their gastric cancer genetic engineered mouse model and cite the relevant papers: PMID: 22001866, 27246044, 31233898. It is possible the metaplasia caused by Tam helps give a headstart to the lesions/tumors.

Response➔We previously demonstrated that blockade of TGFβ signaling in damaged and regenerative colonic mucosa of DSS-treated mice promotes the development of invasive tumors (Oshima et al, Cancer Res, 2015, *Ref. 30*). As Reviewer #2 pointed out, it is therefore possible that tamoxifen-induced epithelial injury in the gastric mucosa and subsequent regenerative responses contributed to the acquisition of invasive potential in gastric tumor cells harboring *Tgfbr2* mutations. In response, we have added this discussion in the revised manuscript (**page 14**), including citation of one of the suggested references (Huh et al, Gastroenterology, 2012, *Ref. 31*).

2. How did the authors activate CreERT2 in vitro? Tamoxifen is usually not miscible in tissue culture media, so most people use 4-hydroxy tamoxifen instead of tamoxifen.
Response➔ We used 4-hydroxy tamoxifen to induce KTP mutations in the established gastric organoid cell lines. We apologize for lack of this information in the original Method section. The relevant details have now added the relevant details to the revised manuscript. (**page 19**).

3. In Figure 1A, authors should clarify the time needed for Tam to induce KTP mutations in organoids. Also, “KP” is an error, as it is the same as “KT”.
Response➔ Mice were administered tamoxifen intraperitoneally (*i.p.*) at 4 mg/mouse at 6 weeks of age, and tumor phenotypes in the glandular stomach were evaluated 28 days after administration. Organoids were treated with 1 μM of 4-OH tamoxifen for 14 days to induce KTP mutations. These tamoxifen treatment schedules for both mice and organoids have been added to in the Method section (**page 17, 19**). In addition, the labeling error in the Figure 1a has been corrected as pointed out by the Reviewer #2.

4. For the invasive lesions in Fig. 1, how confident are the authors that the glandular forms found, especially solitary ones are actually invasive? Because when there is marked hyperplasia of mouse stomach, the tissue can be distorted such that glands can twist around and be cut in cross-section, appearing pseudo-invasive. Although the image is small, it does look as though the single gland invasion in 1G is invasive, because there is distortion of surrounding stromal cells, and there seems to be an actual tumor (mass) formed, but this call can be tricky in many cases.
Response➔To distinguish pseudo-invasion, such as glandular herniation, from true tumor invasion, we followed the criteria described for mouse intestinal tumors (Boivin et al, Gastroenterology, 124: 762-777, 2003). Specifically, in the submucosa of mice carrying KT mutations (i.e., KT, KTP, WKT, and WKTP), we observed invasive tumors

characterized by irregular or angulated glands with dysplastic features, accompanied by desmoplastic stromal reactions (Fig. 1g top and Fig. 1h). However, as Reviewer #2 pointed out, the single round-shaped gland (Fig. 1g top left in the original manuscript) might represent herniation of a hyperplastic gland rather than true tumor invasion. Accordingly, we have removed the data corresponding to this "*single gland invasion*" from the original Fig. 1g. This modification does not affect our conclusion that KT mutations promote the invasive phenotype. To more clearly illustrate the dysplastic features of the invasive tumors, we have added higher-magnification images of E-cadherin IHC in the insets in **Figure 1g**. Figure legend has been updated accordingly.

5. There seems to be a loss of Muc5AC-positivity in the pit zone of Wnt-expressing tumor mice. What makes up the pit in these mice? Is there a reason why overexpression of Wnt would block foveolar cell differentiation?

Response➔As suggested by Reviewer#2, differentiation of gastric epithelial cells appears to be suppressed in *Wnt1*-expressing compound mutant mice (WKT, WKP, and WKTP), as most epithelial cells are proliferative, and Muc5AC-positive foveolar cells as well as H+/K+ ATPase-positive parietal cells are absent. However, such changes were not observed in the stomachs of simple *Wnt1* transgenic mice. Therefore, it is possible that the combination of Wnt activation and Kras mutation leads to suppression of gastric epithelial differentiation and promotes the formation of dysplastic tumors. We have added this comment to the Results section (**page 6**).

6. The mouse genetics are a bit unclear. Is the Trp53R270H allele kept as a heterozygote with the other, endogenous Trp53 allele wild-type? Presumably, the Kras gain of function is an LSL?

Response➔We apologize for not providing a detailed information regarding the mouse genotypes. We have now included the precise description of mouse genotypes in the Method section as *Tgfbr2*$^{flox/flox}$, *Trp53*$^{+/LSL\text{-}R270H}$ and *Kras*$^{+/LSL\text{-}G12D}$ mice (**page 17**). In addition, we have provided the corresponding genotypes in the Results section as follows: K (*Kras*$^{+/G12D}$), KT (*Kras*$^{+/G12D}$, *Tgfbr2*$^{-/-}$), KP (*Kras*$^{+/G12D}$, *Trp53*$^{+/R270H}$), and KTP (*Kras*$^{+/G12D}$, *Tgfbr2*$^{-/-}$, *Trp53*$^{+/R270H}$) (**page 5**).

7. Figure 2C is missing the statistical analysis.

Response➔We have added the results of statistical analyses in **Figure 2c** and confirmed a significant decrease in proliferation of WRN-0% KTP cells compared to WRN-50% KTP cells. A significant reduction was also confirmed in WRN-0% + C59

(PORCN inhibitor) WKTP cells compared to WRN-0% WKTP cells. Figure legend has been updated accordingly.

8. Figure 3 and Figure 4: for the KTP/WKTP/Apc-KTP/Wnt-KTP spleen injection experiment, besides in the liver, is there any other organ metastasis? Especially in the WKTP group, the liver metastasis phenotype is very strong as shown in Figure 4F.
A: typo, “speen” should be “spleen”.

Response➔Based on routine pathological examination, no metastatic lesions were detected outside the liver in any of the spleen transplantation models, including those with WKTP cells. We have added this clarification to the Results section (**page 8**). We apologize for a typographical error in Figure 3a and have corrected “speen” to “spleen”.

9. Figure 3F: needs to show the representative H&E images of tumor area/field. Moreover, how are we to interpret reproducibility/statistics here? One control mouse is lower than all 3 in GNF. Are the datapoints means from many tumor areas/fields in a single mouse? There is no stat analysis here either.

Response➔To show representative H&E images of tumor area/fields, we have added low-magnification H&E images of liver tissues from both control and ETC-159-treated mice in **Figure 3g** (left). These images illustrate that the tumor area/field was markedly reduced following ETC-159 treatment.

As the reviewer pointed out, each datapoint in the graph represents the mean tumor area/field per mouse. In response to this comment, we increased the number of mice in each experimental group to n=8 for control, n=5 for ETC-159 treatment, and n=5 for GNF-6231 treatment, and performed statistical analysis. As shown in the revised **Figure 3f** (right), ETC-159 treatment significantly suppressed liver metastasis of WKTP organoid cells compared to control. GNF-6231 treatment also resulted in a reduction in tumor aria/field, although the difference was not statistically significant. Given the variability in tumor area/field observed among control mice, we concluded that Wnt ligand expression is critical for the development of liver metastasis in this model of gastric cancer. These new data have been included in **Figure 3f** (right) and described in the Results section (**page 8**). Figure legend has been updated accordingly.

10. Figure 3H: authors used GFP to trace tumor cells. But where is the GFP coming from? Authors did not mention the use of GFP in the mouse strains. Authors should

clarify all the detailed genotypes of the mouse strains used in current manuscript (W, K, KT, KP, KTGP, WKT, WKP, WKTP).

Response➔We apologize for not providing information regarding the GFP-expressing tumor organoids. GFP-expressing KTP and WKTP organoids were generated by transfecting a GFP expression vector. We have now added a description of the GFP-labeling procedure in the Method section (**page 19**) and clarified that GFP-labeled organoids were used in the relevant experiments in the Results section (**page 8**).

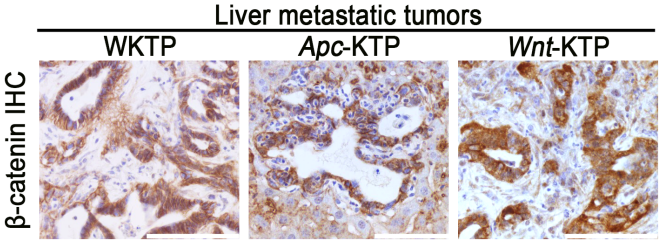
In accordance with the reviewer's suggestion, we have also provided detailed genotypes of all mouse strains used in this study, including W, K, KT, KP, KTP, WKT, WKP, WKTP, in **Supplementary Fig. 2**.

11. Figure 4C needs to label the protein size (kDa). Why not western blot for APC and Wnt1?

Response➔We have added molecular weight markers (kDa) to the immunoblotting data in **Figure 4c**. Detection of APC protein by Western blotting is technically challenging due to its high molecular weight and poor transfer efficiency to the membrane. Additionally, most Wnt ligands are difficult to detect by Western blotting due to the lack of reliable antibodies. Therefore, we assessed Wnt pathway activity by detecting the active form of β-catenin via immunoblotting and by performing the TOPFLASH reporter assay. These approaches are widely used and considered reliable for evaluating Wnt signaling activity.

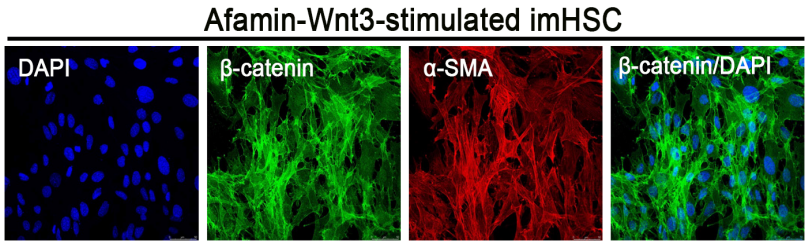
12. Figure 4F/Supplementary Figure 5B experiment need the β-catenin IHC staining results. Are there more nuclear β-catenin in stromal cells in the Wnt-KTP group compared to KTP or Apc-KTP groups?

Response➔In response to the reviewer's suggestion, we performed immunohistochemistry (IHC) for β-catenin in liver metastatic lesions derived from WKTP, Apc-KTP, and Wnt-KTP cells. Accumulation of β-catenin was observed in tumor epithelial cells across all groups; however, we did not detect nuclear β-catenin accumulation in the stromal compartment (See figure below).



We also performed fluorescent immunocytochemistry for β-catenin in Afamin-Wnt3-stimulated hepatic stellate cells (imHSCs). Although increased TOPFLASH activity and induction of Wnt-target genes were confirmed in Wnt3-stimulated imHSCs (Fig. 6b, c), nuclear β-catenin accumulation was not observed in these cells (See figure below).

These findings suggest that Wnt-activated stromal fibroblasts may not accumulate nuclear β-catenin to a level detectable by conventional IHC or ICC, unlike tumor epithelial cells. Nonetheless, considering potential technical limitations in detecting nuclear β-catenin in fibroblasts, we have chosen not to further discuss this point in the manuscript. We hope that the reviewer understand our decision.



13. The labels among the groups in Figure 4F and Figure 4G are confusing. There is one group of KTP mice in Figure 4F, but with two columns for KTP mice (#1,2) shown in Figure 4G; and one group of WKTP mice in Figure 4F but with three columns shown in Figure 4G (#1,2,3). In Figure 4F, the KTP and WKTP image section is missing a scale bar.

Response➔We apologize for confusing presentation in the original Figure 4f and 4g. We examined two KTP organoid lines (#1 and #2) and three WKTP organoid lines (#1, #2, and #3) in spleen transplantation experiments, as shown in Figure 4g and Supplementary Figure 6c. In the original Figure 4f, we showed representative liver tissue photographs of one KTP line (KTP#1) and one WKTP line (WKTP#2) without indicating the organoid line numbers, which may have caused confusion. To clarify this, we have now added the organoid line numbers for both KTP and WKTP samples in Figure 4f. Additionally, to illustrate the heterogeneity in tumor phenotypes among the WKTP lines, we have added liver photographs from WKTP#1 in **Figure 4f**. Figure legend has been updated accordingly.

We also apologize for missing a scale bar in the Figure 4f image; this has now been corrected.

14. The authors should perform a transwell assay to test the cell invasion ability among KTP, WKTP, Apc-KTP, and Wnt-KTP groups.

Response➔As suggested by the reviewer, we have performed transwell assay using KTP, WKTP, Apc-KTP, and Wnt-KTP organoid cells to assess their invasive potential. We found no significant differences in invasiveness among these four organoid lines. These data have been added to **Supplementary Fig. 6a and 6b**, and described in the Results section (**page 9**).

15. Supplementary Figure 5A is missing Wnt-KTP cell data. How did authors reach the conclusion that "the proliferation rates of Apc-KTP and Wnt-KTP cells were unchanged compared to parental KTP cells"?

Response➔We have added representative culture images and CTG assay results for *Wnt*-KTP cells in **Supplementary Fig. 6a and 6b** (original Supplementary Fig. 5a). Based on these new data, we concluded that the proliferation rate of *Apc*-KTP and *Wnt*-KTP cells are comparable to those of parental KTP cells. This conclusion has been indicated in the Results section (**page 9**).

16. Figure 5B, 5D and Supplementary Figure 6 are missing a scale bar.

Response➔We apologize for the absence of scale bars in Figure 5b, 5d, and Supplementary Figure 7 (original Supplementary Fig. 6). We have now added scale bars in these images.

17. "Hepatocyte marker Alb was significantly increased in cluster 3 and 6, while epithelial markers, Ceacam1 and Muc1, were increased…" Technically, liver is epithelium also, so this should be clarified, because it is not clear which epithelial markers were used or why the ones shown are the representative epithelial cell markers.

Response➔As Reviewer#2 correctly pointed out, hepatocytes are epithelial cells; therefore, it was inappropriate to use the term "epithelial markers" to distinguish gastric tumor cells (WKTP cells) from hepatocytes. Initially, we referred to the PanglaoDB public database and selected Ceacam1 and Muc1 from the list of "epithelial markers".

For the revision, we reanalyzed the mRNA expression levels of *Ceacam1*, *Muc1*, and *Alb* in WKTP cells and normal liver tissue by RT-PCR. Among them, *Muc1* showed significantly higher expression in WKTP cells, while *Ceacam1* did not. In contrast, *Alb* expression was significantly elevated in liver tissues. To further refine the distinction, we searched for additional WKTP-specific marker genes using database analysis and

confirmed by RT-PCR that *Prom1* expression is markedly increased in WKTP cells compared to hepatocytes. Accordingly, in the revised manuscript, we replaced the term “epithelial markers” in the original Figure5c, and now present violin plots of *Muc1* and *Prom1* as “WKTP cell markers” in the updated **Figure 5c**. These findings are described in Results section (**page 10**). RT-PCR data for *Muc1*, *Prom1*, and *Alb* have also been added to **Figure 5c**. Figure legend has been updated accordingly.

18. The authors should show representative IHC image results (translational level) to validate the spatial transcriptomic data (transcriptional level), for CTNNB1, WNT3A, TGFB1, SAMD3, and SMAD4.

Response➔The results shown in Figure 5e indicate activation of Wnt signaling and TGFβ pathways based on IPA analysis using differentially expressed genes in cluster 5. However, this does not necessarily imply that individual pathway components─such as *Ctnnb1*, *Wnt3A*, *Tgfb1*, *Smad3*, and *Smad4*─are transcriptionally upregulated.

As Reviewer #2 correctly pointed out, validation of the spatial transcriptomic data at the protein level is important. However, as noted in our response to comment #12, detecting nuclear β-catenin in fibroblasts by IHC is technically challenging. Instead, Wnt signaling activation in HSCs and CAFs was confirmed through in vitro experiments (Figure 6).

In contrast, we were able to detect phosphorylated Smad2 (P-Smad2) by IHC in fibroblastic cells within the metastatic tumor stroma, indicating activation of TGFβ pathway. These IHC results for P-Smad2 have been added to **Supplementary Figure 7b** and described in the Results section (**page 11**).

19. In Figure 7A, Has2 is not limited to cluster 5, which raises the question of whether the authors have performed the filtering before analyzing the spatial transcriptomic data by using Loupe Browser? The authors did not mention how they processed the spatial transcriptomic data. This should be detailed.

Response➔*Has2* expression is significantly enriched in cluster 5, as shown in the violin plot in Figure 7a and in Supplementary Table1. However, as the reviewer pointed out, its expression is also detected at lower levels in other clusters. In the VISIUM platform, each spot with 55 μm in diameter contains more than 10 cells, which may account for the presence of lower *Has2* expression in other clusters that include stromal cells.

We apologize for not providing sufficient methodological details regarding spatial transcriptomic data processing in the original manuscript. Raw spatial transcriptomics

data were processed using the Space Ranger pipeline (v2.0.1, 10x Genomics, Pleasanton, CA), aligned to the mm10-2020-A mouse reference genome, and analyzed from the resulting FASTQ files. For downstream analysis, including UMAP visualization, violin plots, and gene expression analysis, we used Loupe Browser v6.0 (10x Genomics). Principal component analysis (PCA) was performed using 100 dimensions. These methodological details have now been included in the Methods section (**page 24-25**).

20. The authors mention HA interacting with CD44 on stem cells and hypothesize the same sort of interaction in gastric cancer, but there is a paper that does show that metaplasia in stomach expands CD44+ progenitor cells that as HA expands, so this paper should be cited and potentially discussed: PMID:23589310. There are other papers detailing CD44 role in metaplasia and stemness in stomach (eg, PMID: 31071489), so the CD44/xCT/HA pathway may be important in both metaplasia and metastasis.

Response➔As pointed out by Reviewer #2, the hyaluronan receptor CD44 has been shown to play a critical role in the proliferation of metaplastic gastric epithelial progenitor cells (Khurana et al, JBC, 2013, *Ref. 37*). In addition, CD44 and xCT are required for the reprogramming of gastric epithelial cells following mucosal injury (Meyer et al, Cell Mol Gastroenterol Hepatol, 2019, *Ref. 38*). Accordingly, we have cited these studies and discussed the possibility that hyaluronan may enhance the stem-like properties of gastric cancer cells through CD44-mediated signaling, thereby contributing to metastatic tumor formation. This discussion has been added in the Discussion section (**page 16**).

21. A visualization of cell-cell communication, using differentially expressed genes (DEGs), would greatly improve analysis, as one would expect important cell-cell communication between cluster 5 and the rest of the clusters. For example, as the authors state, CD44 in tumor cells could be communicating with HA in stroma.

Response➔In this study, we identified that tumor cell-derived Wnt ligands activate Wnt signaling in surrounding stromal cells and induce *Has2* expression in cooperation with TGFβ signaling. Furthermore, we demonstrated that hyaluronan (HA) deposition in the tumor stroma is critical for the development of liver metastatic tumors.

As suggested by Reviewer #2, cell-cell communication analysis using spatial transcriptomic data could provide important mechanistic insights into HA-associated metastasis development. While we agree that this approach is highly valuable, we

believe that such analyses are beyond the scope of the present study. Nonetheless, we greatly appreciate the reviewer's constructive suggestion and intend to pursue this line of investigation in future study.

Reviewer #3 (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.

Reviewer #4 (Remarks to the Author)

.....The authors concluded that paracrine Wnt signaling activation in hepatic stellate cells in conjunction with TGFβ results in increased Has2 expression and this promotes metastatic tumor development through hyaluronan deposition and increased ECM production. This study follows an earlier one by the same group where the role of these driver mutations in formation of intestinal tumors was analyzed (Kok et al, 2021).

A similar approach to generating gastric cancer GEMMs was published by Seidlitz T. et al in 2019 so the strategy used is not entirely novel. However, the present study adds novelty, by identifying a major role for Wnt ligands in shaping the liver microenvironment and thereby, enabling gastric cancer liver metastases.

Technically, the experiments were well executed, and the manuscript is (with a few exceptions) well written. However, there are several issues that need to be addressed

Response➔We sincerely thank Reviewer #4 for the constructive comments on our manuscript. We have carefully addressed all the comments as detailed below.

General comment:

In Seidlitz et al, a similarly constructed GEMM based on driver mutations KrasG12D/+;Tp53R172H/+;Smad4fl/f in the gastric epithelium resulted in gastric tumors that were metastatic to liver and lung even in the (apparent) absence of Wnt production. These findings are at variance with the results obtained in the present study with the KTP mice and the authors should discuss potential differences that could explain the different phenotypes.

Response➔As the reviewer correctly pointed out, the GEMM model used by Seidlitz

et al, (*Ref. 16*), which harbors *Kras*^{G12D}, *Smad4*$^{-/-}$, *Trp53*^{R172H} mutations, developed gastric cancer with liver metastasis despite in the apparent absence of transgenic Wnt ligand expression. Several key differences between their model and ours (KTP mice) may account for the distinct metastatic phenotypes.

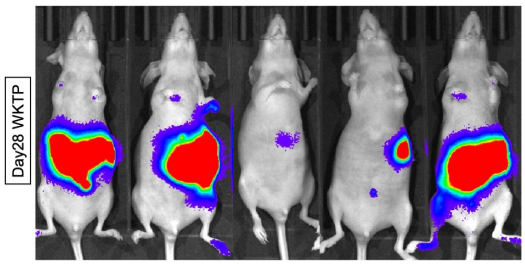
First, the CreER drivers used to induce recombination may target different epithelial cell populations: *Anxa10*-CreER in the Seidlitz model versus *Claudin18*-CreER in our model. These possible target cell differences could influence the tumorigenicity and metastatic potential of the resulting tumors.

Second, the specific mutations in the TGFβ pathway and p53 differ: Seidlitz et al. used *Smad4* deletion and *Trp53*^{R172H}, while our model includes *Tgfbr2* deletion and *Trp53*^{R270H}. These molecular differences may affect the regulation of Wnt signaling in tumor cells. We have briefly discussed these points in the Discussion section (**page 15**).

Specific points

1. Figure 2 (E-H). This model of orthotopic implantation best mimics the clinical pathology. Did the authors analyze progression to metastatic disease in these mice? Do WKTP tumors spontaneously metastasize to the liver in this setting? If not, a discussion is warranted.

Response➔ We thank the reviewer for this important comment. In our orthotopic transplantation experiments, we monitored gastric tumor development using in vivo imaging up to day 21, as shown in Figure 2g. At this time point, no clear evidence of metastasis was observed. However, by day 28, some mice began to exhibit signs of peritoneal dissemination, as demonstrated in the imaging data provided below. Although histological analysis at this stage did not reveal liver metastases, the mice showed signs of clinical deterioration, limiting our ability to extend observation further. Thus, we could not examine whether WKTP tumors spontaneously metastasize by long term analysis.



To establish a more quantitative and reproducible model for assessing liver metastasis, we therefore employed the intrasplenic injection approach in this study. Although this model bypasses early steps of the metastatic cascade, it enables robust evaluation of the role of Wnt ligands in supporting metastatic outgrowth in the liver. To clarify this point in the manuscript, we have revised the sentence in the Results section (**page 7**) as follows: “To investigate

the role of ligand-dependent Wnt signaling in liver metastasis by disseminated tumor cells, we transplanted KTP and WKTP organoid cells into the spleen of mice and analyzed liver tissues."

2. Figure 3F - Were the differences between the different group statistically significant? If not, this should be stated. There appears to be a high variability in the data obtained for the control group and analysis of additional mice may be necessary to support the conclusion that these inhibitors had an effect.

Response➔In response to this comment, we increased the number of mice for each experiment group to n=8 for control, n=5 for ETC-159 treatment, and n=5 for GNF-6231 treatment, and performed statistical analysis. As shown in the revised Figure 3f, ETC-159 treatment significantly suppressed liver metastasis of WKTP organoid cells compared to the control. GNF-6231 treatment also resulted in a reduction in tumor aria/field, although the difference was not statistically significant. Given the variability in tumor area/filed observed among control mice, we concluded that Wnt ligand expression is critical for the development of liver metastasis in this model of gastric cancer. These new data have been included in **Figure 3f**, and described in the Results section (**page 8**).

3. Figure 3 (H&I) – Among the earliest events in liver metastases formation is the recruitment of innate immune cells, among them neutrophils and monocytes which are still functional in NSG mice. Wnt1 is known to affect immune cell function including macrophage polarization (e.g. Staal et al, 2008, Haseeb M et al 2109). Given the results shown this figure, the authors should analyze or at least comment on the potential role of myeloid-derived cells in promoting establishment of metastases of WKPT cells.

Response➔As the reviewer suggested, we performed immunohistochemistry for αSMA and F4/80 to detect activated fibroblasts and macrophages, respectively, using liver sections at day 3 and 7 after spleen transplantation of KPT or WKTP cells. Notably, we observed fibroblast activation and macrophages infiltration in the stroma of WKTP micro-metastatic tumors at day 7 WKTP. These IHC results have been added to **Supplementary Figure 4** and described in the Results section (**page 8**). As the reviewer suggested, it has been reported that Wnt/β-catenin signaling promotes M2 polarization of tumor-associated macrophages (TAMs) (Tigue et al, Cancer Res, 2023, *Ref. 32*). We have now discussed the potential role of Wnt-induced M2 TAMs in facilitating metastasis development in the Discussion section (**page 14, 15**).

4. Fig 4C- The ratio of activated:total beta-catenin appears to be higher in KTP than in WKTP cells. This is surprising. Is there no autocrine effect of Wnt1 in the WKTP cells? Does Wnt1 only act in a paracrine fashion and specifically only in the liver? Did the authors quantify Wnt1 production levels in the three models (KPT, WKPT and Wnt-KPT)? These data are important for interpretation of the results.

Response➔We quantified the band intensity of active β-catenin in our immunoblotting data using ImageJ and normalized the values to GAPDH levels. While active β-catenin levels in KTP cells were not higher than those in WKTP cells, they were rather comparable. These results are presented as bar graph in **Figure 4c**. In contrast, TOPFLASH reporter assay (Figure 4d) demonstrated that Wnt signaling activity is significantly higher in WKTP cells compared to KTP cells. These data suggest that Wnt1 ligands may activate Wnt signaling in WKTP cells via an autocrine mechanism, without leading to a detectable increase in active β-catenin levels by immunoblotting.

We previously observed similar results in RNF43-mutant (Wnt ligand-dependent) colon cancer cells, where exogenous Wnt ligands activated Wnt signaling without significantly increasing active β-catenin levels. Therefore, in response to the Reviewer's question, we believe that WKTP cells utilize self-secreted Wnt1 ligands to support their survival and proliferation, as supported by the data shown in Figure 2b and 2C.

Regarding Wnt1 expression, Wnt ligands undergo palmitoleoylation by PORCN in the endoplasmic reticulum, which is essential for their secretion. Therefore, to accurately compare the levels of biologically active Wnt1, it is necessary to measure secreted Wnt1. However, due to the lack of reliable antibodies suitable for quantification by ELISA or Western blotting, this is currently not feasible. Importantly, our TOPFLASH reporter assay (Figure 4d) showed that Wnt signaling activities in WKTP and *Wnt*-KTP cells are significantly higher than that in KTP cells.

5. Figure 4F- How do the authors explain the striking difference in the extent of liver metastatic disease in WKPT and Wnt-KPT implanted mice? Were Wnt1 production levels in these cells compared?

Response➔As shown in the bar graph of Figure 4g, there is variation in tumor area per field among the three independently established WKTP organoid lines, and even among replicates of the same line. In the original Figure 4f, we presented liver metastasis images from the WKTP#2 line, which exhibited a markedly aggressive phenotype compared to Wnt-KTP, as the Reviewer pointed out. However, the levels of

tumor area per field for the WKTP#1 line was comparable to that of *Wnt*-KTP organoids (Figure 4g). Therefore, the striking difference in liver metastasis observed between WKTP#2 and *Wnt*-KTP may reflect biological variability among organoid lines with the same genotype. To address this, we have added representative images of liver tumors derived from WKTP#1 in addition to WKTP#2 in the revised **Figure 4f**.

Regarding the detection of Wnt1 expression, we have responded to the comment#4. TOPFLASH reporter assay (Figure 4d) showed no significant difference in Wnt signaling activity between WKTP and *Wnt*-KTP cells.

6. Figure 5- To determine whether the extensive stromal response observed in these livers is a cause or consequence of metastatic expansion, livers should also be analyzed at earlier time points. It is also of interest to know whether the spatial transcriptomic analysis identified any myeloid/innate immune cell clusters and whether liver sections of KPT -implanted mice were also analyzed for comparison.

Response➔Determining whether the formation of a fibrotic microenvironment in the liver is a *cause* or *consequence* of metastatic colonization is one of the major goals of our research. In our previous study (Kok et al., *Nat Commun*, 2021, *Ref. 19*), we demonstrated that metastasis was not developed when host TGFβ signaling was genetically disrupted, as the stromal response was not induced even after dissemination of metastatic tumor cells. In the present study, we also showed that hyaluronan production in the stroma is essential for metastatic tumor formation. Taken together, these findings suggest that the stromal activation in the liver is a prerequisite for the development of metastatic lesions. In the conclusion of the current manuscript, we have highlighted the therapeutic potential of targeting CAF-mediated Wnt signaling and Has2 expression in the tumor stroma (**page 16**).

In line with Reviewer Comment #3, we performed immunohistochemical analyses of liver tissues at days 3 and 7 after splenic transplantation of organoids. These analyses revealed that prominent infiltration of F4/80-positive macrophages in the stroma of WKTP cell-derived tumors, whereas such infiltration was less evident in KTP-derived lesions. Given that Wnt/β-catenin signaling has been reported to promote M2 polarization of TAMs, it is plausible that Wnt-induced M2 TAMs may contribute to metastatic progression. This possibility is now added in the Discussion section (**page 14**). We plan to perform spatial transcriptomic analyses as part of our future investigations. We hope the reviewer understands our rationale.

7. Discussion and data interpretation

The authors propose that the ability of KPT and WKPT organoid to form tumors and grow equally well in the orthotopic (and subcutaneous) site is due to local Wnt1 production by stromal cells. This is speculative and should be experimentally tested, for example by using the PORCN Wnt1 inhibitor C59. This also raises the question why the authors switched from an orthotopic to an intra-splenic model to evaluate liver metastasis. If WKPT cells do not metastasize following intra-mucosal implantation (or metastasis is a very late event) this should be stated and discussed.

Response➔To address this comment, we performed subcutaneous transplantation of KTP organoids and treated the recipient mice with the PORCN inhibitor ETC-159. While ETC-159 treatment significantly suppressed liver metastasis formation of WKPT cells (Figure 3f), it did not inhibit subcutaneous tumor development. Although it is possible that KTP cells do not require exogenous Wnt ligands for proliferation in the submucosal and subcutaneous environments, we currently lack direct mechanistic data to support this hypothesis. Therefore, we have softened the wording in the Results section (**page 7**) to: “These results suggest that the combination of KTP driver mutations is sufficient to induce dysplastic tumor formation in both the submucosal and subcutaneous environments.”

Although our current experiments do not directly test stromal Wnt ligand dependency in the subcutaneous setting, this does not impact the main conclusions of our study.

Regarding the metastasis analysis in orthotopic models, we have described about limitation of orthotopic models for long term analysis in our response to the Comment#1.

8. The functional significance of hyaluronan in metastatic expansion in the liver in this model has not been defined. Is it utilized by WKPT cells to attach via CD44? Whether the fibrotic response is a cause or consequence of tumor expansion in the liver should be resolved experimentally by analyzing the response on day 7 not at late stages of metastasis as demonstrated in Fig 5. Language editing is required. For example see line 329.

Response➔As the reviewer pointed out, hyaluronan receptor CD44 plays a key role in the proliferation of gastric progenitor cells (Khurana et al, JBC, 2013, *Ref. 37*). Furthermore, CD44 and xCT have been shown to promote the reprogramming of gastric epithelial cells (Meyer et al, Cell Mol Gastroenterol Hepatol, 2019, *Re. 38*). These findings suggest that hyaluronan may enhance the stem-like properties of

gastric cancer cells via CD44-mediated signaling, thereby contributing to metastatic tumor formation. We have included this point in the Discussion section (**page 16**).

Additionally, it has been reported that low molecular weight (LMW)-hyaluronan promotes tumor progression, whereas high molecular weight (HMW)-hyaluronan exerts tumor-suppressive effects (Liu et al, Front Immunol, 2019, *Ref. 35*). Moreover, a recent study showed that reducing LMW-hyaluronan levels with hyaluronidase inhibitor suppresses cancer cell metastasis (McGuire et al, Sci Rep, 2024, *Ref. 36*). These results, together with our findings, support the notion that LMW-hyaluronan plays a critical role in promoting gastric cancer metastasis. We have discussed this aspect in the Discussion section (**page 15-16**).

Regarding whether the fibrotic niche is a cause or consequence of tumor expansion in the liver, our data shown in the Supplementary Figure 4 demonstrate that a fibrotic microenvironment marked by αSMA+ fibroblasts is already established at day 7 in WKTP cell micrometastasis, while such a fibrotic reaction is absent in KTP cell clusters. These results suggest that fibrotic niche formation is a consequence of WKTP cell colonization. On the other hand, degradation of stroma cell-derived hyaluronan significantly suppressed development of metastasis (Figure 7f, g), indicating that stromal remodeling is a driving factor in tumor progression. Thus, stromal activation may act both as a cause and consequence of metastatic growth.

We acknowledge that the interaction between disseminated tumor cells and host stromal responses is complex. Therefore, we plan to conduct more detailed mechanistic studies in future projects to further elucidate this relationship.

We have carefully revised the manuscript to improve English usage throughout, including the point noted by the reviewer (line 329 in original manuscript).

Reviewer #5 (Remarks to the Author)

General Remark: While the study is interesting, there is very little evidence that the findings could be translated to human (very little has been done to validate the findings in human). While the study uses an experimentally sophisticated system (many transgenic animals) there is an important number of issues that needs to be carefully addressed. When talking about Wnt-signaling and paracrine communication between cancer cells and CAF it is important to note that specific Wnt ligands will be secreted by specific cells while specific receptors will be expressed on certain cells. This is where the study in reviewers opinion is limited and to certain extent far from realistic situation encountered in human.

Response➔We appreciate the reviewer's insightful comment. We fully agree that it is important to clarify which specific Wnt ligands and Frizzled receptors mediate metastatic progression in human gastric cancer. However, the current understanding of the pairing between the 19 Wnt ligands and 10 Frizzled receptors remains incomplete. Notably, Nanki et al. (Cell, 2018, *Ref. 10*) reported substantial heterogeneity among gastric cancer organoids derived from 36 patients, particularly regarding their dependency on exogenous Wnt ligands or R-spondin, as well as endogenous Wnt ligand expression patterns by the cancer cells themselves. Furthermore, TCGA data shows that 18 Wnt ligands are each upregulated in more than 10% of gastric cancer samples, and that such Wnt ligand upregulation is found in approximately 85% of cases (Supplementary Fig. 1), suggesting that multiple Wnt ligands may be functionally relevant. Thus, whether specific ligand–receptor pairs are dominant, or if redundant signaling mechanisms exist in gastric cancer, remains an open question likely requiring large-scale single-cell transcriptomic analysis.

Our current study demonstrates that Wnt ligand-mediated signaling promotes the development of metastatic lesions in gastric cancer. While we acknowledge that further investigation is necessary to define the precise ligand–receptor combinations involved in human metastasis, we believe that our findings provide important insights and a strong foundation for such future research. We hope the reviewer understands the scope and significance of our present work in this context.

Major Remark 1: In human gastric cancer HAS2 is not expressed on stellate cell derived CAF. Namely, there are generally 2 types of CAF (in the liver) depending on their origin, stellate cell derived (RGS5+) or resident fibroblast derived (PDGFRA+). Hence the model that is used in vitro (employing immortalised HSC) is unfortunately irrelevant. This goes further, HYAL2 is not expressed by cancer cells, but by endothelial cells while Wnt5A is expressed by cancer cells.

I am attaching in support some meta-analysis that I have conducted based on previously published data (DOI:10.1002/ctm2.730)

In conclusion, the authors need to be very careful about which ligand is expressed on which cell (lay out their model based on human data, either single cell if clear enough or IHC). Then according to this design their experimental strategy. Concretely, which cells express WNT1 in human gastric cancer and what is the target receptor(s), where are these receptors situated in humans?

Response➔Thank you for your important comment regarding the origin of CAFs. As cited by the reviewer, CAFs in gastric cancer can be categorized into two major

populations: iCAFs (expressing PDGFRA and CXCL12) and mCAFs (myCAFs) (expressing RGS5 and ACTA2). GSEA results suggest that iCAFs primarily promote stromal remodeling and cancer cell proliferation and invasion. We fully understand the reviewer's concern that using HSCs for in vitro experiments may not completely reflect the functional CAF populations found in human gastric cancer.

However, the origin and classification of CAFs within the tumor stroma remain controversial. For example, a recent review by Geng and Schwabe (Hepatology, 2025, *Ref. 23*) indicates that HSCs can differentiate into both iCAFs and myCAFs. Moreover, myCAFs expressing *HAS2* have been reported to promote tumor progression through hyaluronan-mediated signaling (Cogliati et al., Nat Rev Gastroenterol Hepatol, 2023). These findings support the relevance of using HSCs for in vitro studies, especially in the context of HAS2-driven metastatic promotion by myCAFs.

As the reviewer rightly pointed out, multiple cellular origins of CAFs likely exist beyond HSCs. To address this, we conducted additional experiments using a CAF line isolated from liver metastatic tumors. These data demonstrate that Wnt ligands activate canonical Wnt signaling also in CAFs, and that Wnt ligands synergize with TGF-β to induce *Has2* expression in CAFs. These new findings have been included in the revised **Figures 6b and 7b**. Figure legends have been updated accordingly.

Regarding HYAL1 and HYAL2, we transfected expression vectors for these genes into WKTP cells specifically to degrade stromal hyaluronan in order to evaluate its functional role in metastasis. We did not assume that HYAL1 or HYAL2 are endogenously expressed by cancer cells *in vivo*.

Concerning the reviewer's question on Wnt ligand and Frizzled receptor expression, we have addressed this point in our reply to the General Remark from the same reviewer. Briefly, our study focuses on how exogenous Wnt ligands, expressed by tumor cells, influence the surrounding tumor microenvironment during metastasis. In our model, Wnt ligands are expressed by cancer cells, and the corresponding Frizzled receptors for Has2 induction are thought to express on CAFs. We selected WNT1 based on previous reports demonstrating its association with gastric cancer aggressiveness and metastasis (Mao et al., Cell Death Dis, 2014, *Ref. 18*; Li et al., Lab Invest, 2023, *Ref. 12*). We have included this rationale for use of Wnt1 in the Results section, along with newly added reference (**page 5**).

Major Remark 2: The part of the data that is related to CAF needs thorough revision. The authors state "It has been shown that CAFs in the liver metastasis are originated from activated hepatic stellate cells (HSCs)", which is simply wrong in many aspects

(the cited literatures is not relevant, please see doi: 10.1038/s41586-019-1631-3.). Again, CAFs in the liver metastasis are derived from at least 3 different precursor cells - and in contrast to what the authors are claiming, those CAF that reorganise the ECM are not stellate cell derived. I further refer to the attached meta-analysis where on primary gastric cancer samples from human we can clearly see at least 2 groups of CAF (stellate cells do exist also in stomach and many other organs next to liver) to begin with.

Response➔We appreciate the Reviewer's important comment regarding the origin of CAFs in liver metastases. In the original text, we stated that "CAFs in the liver metastasis are originated from activated hepatic stellate cells (HSCs)." However, as the Reviewer correctly pointed out, CAFs can arise from multiple precursor cell types, and HSCs represent one of several possible sources. We also realized that the cited literatures were not appropriate, and have therefore deleted both the statement and associated references from the manuscript.

In the revised Results section (**page 11**), we now state that CAFs in gastric cancer tissues can be classified into myCAF and iCAF subtypes based on distinct gene expression profiles, as described by Jiang et al. (Clin Transl Med, 2022, *Ref. 21*), as referenced in the Reviewer's comment #1. We also mention that HSCs have the potential to differentiate into both myCAFs and iCAFs. However, consistent with current understanding, we emphasize that CAFs exhibit substantial heterogeneity in their cellular origins, citing relevant literatures including Yamamoto et al. (Cancer Sci., 2022, *Ref. 24*).

In response to the Reviewer's suggestion that the use of only HSCs is insufficient to assess CAF function, we performed additional *in vitro* experiments using a CAF line isolated from liver metastatic tumors. These new results have been incorporated into **Figures 6b and 7b** in the revised manuscript.

Major Remark 3: Provided that the authors sort out the cellular identities according to the remarks made above, what is the link between Wnt-signalling and HAS2 expression? The authors switch without any explanation from Wnt1 (which is their initial model) to Wnt3. What is the reason for this? What is the downstream signalling and how Wnt1 (and not 3) could cause the HAS2 over-expression?

Response➔We appreciate the Reviewer's insightful comment regarding the mechanistic link between Wnt signaling and HAS2 expression, as well as the rationale for using Wnt3 in our in vitro experiments.

In this study, we experimentally demonstrated that Wnt ligands expressed by gastric cancer cells induce *HAS2* expression in CAFs in a manner synergistic with TGF-β signaling. This conclusion is supported by spatial transcriptomic analysis, *in vitro* experiments using HSCs and CAFs, histological analysis of human and mouse gastric cancer liver metastases, and analysis of public databases showing a positive correlation between Wnt ligand and *HAS2* expression (Supplementary Fig. 8).

Regarding the use of Wnt3 instead of Wnt1 in our *in vitro* experiments, we employed afamin-bound Wnt3a because it has been reported to be water-soluble and to provide stable and potent canonical Wnt signaling activity (Mihara et al., eLife, 2016, *Ref. 25*). Since both Wnt1 and Wnt3a activate the canonical Wnt/β-catenin signaling pathway, afamin-Wnt3a serves as a functional surrogate for Wnt1 in our experimental system. To clarify this point, we added the following sentence to the Results section (**page 11**): “we conducted experiments using both HSCs and CAFs, treating them with afamin-bound Wnt3a, which preserves stable canonical Wnt ligand activity (25).”

With respect to downstream signaling, both Wnt1 or Wnt3a bind to Frizzled receptors, leading to inhibition of GSK3-mediated phosphorylation of β-catenin. This results in β-catenin stabilization and nuclear translocation, where it interacts with TCF transcription factors to induce expression of canonical Wnt target genes. Notably, TCF7L2, a key Wnt pathway transcription factor, has been reported to directly bind the *Has2* promoter and induces its expression (Sun et al., Biomolecules, 2022, *Ref. 34*). This provides a possible mechanism by which both Wnt1 and Wnt3a may drive *Has2* expression via canonical Wnt signaling. We have included this mechanistic insight and supporting reference in the revised Discussion section (**page 15**).

Major Remark 4: Hyaluronidase inhibitors have been shown to inhibit cancer metastasis (https://www.nature.com/articles/s41598-024-64924-6). The authors here show the contrary. It seems that high molecular weight hyaluronic acid (HMW-HA) is tumor protective, while low molecular weight hyaluronic acid (LMW HA) is tumor promotive. Authors need to clarify better which type of HA they are dealing with and relate this to what is observed in human samples.

Response➔We appreciate the Reviewer’s insightful comment. As the Reviewer noted, low molecular weight (LMW) hyaluronan has been reported to promote tumor progression, whereas high molecular weight (HMW) hyaluronan exhibits tumor-suppressive effects (Liu et al., Front Immunol, 2019, *Ref. 35*). In the study cited by the Reviewer (McGuire et al., Sci Rep, 2024, *Ref. 36*), treatment with delphinidin, a

potential hyaluronidase inhibitor, led to the accumulation of HMW-hyaluronan and was associated with suppression of cancer invasion and metastasis.

In our study, we simultaneously overexpressed two hyaluronidases, *Hyal1* and *Hyal2*, in WKTP tumor cells. Hyal2 initiates to breakdown of HMW-hyaluronan into LMW-fragments, while Hyal1 further degrades LMW-hyaluronan into smaller oligosaccharides, promoting its clearance (Bourguignon and Flamion, FASEB J, 2016, *Ref. 27*). Thus, in both McGuire et al. and our studies, the level of tumor-promoting LMW-hyaluronan is reduced, albeit through different mechanisms-one by inhibiting its generation, the other by enhancing its degradation and clearance. Therefore, we believe that our findings are not contradictory to those reported in the cited literature.

To clarify this point, we have added the following statement to the Results section (**page 13**): " We anticipated that *Hyal*-WKTP cells, which co-express *Hyal1* and *Hyal2*, would degrade both high molecular weight (HMW)- and low molecular weight (LMW)-hyaluronan within the tumor microenvironment (27)."

Additionally, we have discussed the possibility that LMW-hyaluronan may play an important role in promoting gastric cancer metastasis in the Discussion section, along with newly added references (**page 15-16**).

Response to the reviewer comments

Our point-by-point responses to the comments made by the Reviewers are included below. The comments are quoted "verbatim", followed by our responses.

Reviewer #2 (Remarks to the Author):

The authors have done a very scholarly job of addressing reviewer concerns. Here are some minor comments:

1. It would be better to mention in the manuscript why authors chose to assess Wnt pathway activity by detecting the active form of β-catenin via immunoblotting and by performing the TOPFLASH reporter assay.

➔We thank the reviewer for this helpful suggestion. In the revised manuscript, we now clarify our rationale for using two complementary assays to assess Wnt pathway activity in the text as follows. "Loss of APC suppresses phosphorylation-dependent β-catenin degradation, thereby increasing the unphosphorylated (active) form of β-catenin. The stabilized β-catenin translocates to the nucleus and functions with TCF/LEF to drive Wnt target gene transcription. Consistent with this rationale, immunoblotting showed a significant increase in unphosphorylated β-catenin in *Apc*-KTP cells compared with KTP and WKTP cells (Fig. 4c), and the TOPFlash reporter assay demonstrated significantly higher β-catenin/TCF transcriptional activity in *Apc*-KTP cells, which is comparable to the *Apc*-disrupted intestinal tumor-derived organoids, AKTP (19) (Fig. 4d)" (page 9).

2. In manuscript lines 213–214, the authors stated: "Importantly, all three independently established Apc-KTP organoid cell lines did not develop liver metastasis, and only rare small clusters of tumor cells were detected." However, in the current Figure 4f (Apc-KTP#2), liver metastases are visible at multiple locations: in the first liver (5 o'clock and 11 o'clock), the second liver (1 o'clock), and the third liver (3 o'clock and 11 o'clock), with tumors present at the liver edges. In addition, Supplementary Figure 6C clearly shows liver metastases in the Apc-KTP group. Therefore, the statement in lines 213–214 is inconsistent with the presented data and should be revised.

➔We thank the reviewer for pointing out this inconsistency. We have revised the Results to accurately reflect the data shown in Fig. 4f and Supplementary Fig. 7c (former Supplementary Fig. 6c) as follows. "Among the three independently established *Apc*-KTP organoid lines, only line #2 developed limited, small macroscopic liver metastasis (Fig. 4f, arrowheads, Fig. 4g), whereas other lines did not show grossly detectable liver metastasis. Moreover, histological analysis revealed rare, small clusters of tumor cells in #2 and #8 *Apc*-

KTP-transplanted livers (Supplementary Fig. 7c), which were below the threshold of gross detection" (page 10).

3. The reason for using a splenic transplant (vs true orthotopic) model as well as not using C57/Bl6 mice as hosts might be mentioned in the final text and not just in the response to reviewers, as some readers may also be curious.

➔We thank the Reviewer #2 for this helpful suggestion. We have added an explanation in the Results describing our use of a splenic transplantation model with showing in vivo imaging data (Supplementary Fig. 4), and in the Discussion, our rationale for using NSG rather than C57BL/6 mice as hosts.

Results:

"We next evaluated the role of Wnt ligands in liver metastasis. In the orthotopic transplantation model of WKTP organoids, three of five mice exhibited signs of peritoneal dissemination by day 28, accompanied by clinical deterioration, which limited the observation window for assessing liver metastasis (Supplementary Fig. 4). We therefore performed spleen transplantation of KTP and WKTP organoids in NSG mice to examine the liver metastatic tumors. (Fig. 3a)" (page 7).

Discussion:

"In our previous study, spleen transplantation of intestinal tumor-derived AKTP organoids, harboring mutations in *Apc*, *Kras*, *Tgfbr2*, and *Trp53* into syngeneic C57BL/6 (B6) mice yielded liver metastasis in ~40% of recipients, whereas injection into immunodeficient NSG mice resulted in nearly 100% penetrance (37). This discrepancy is likely due to the NK cell-mediated surveillance in B6 mice, although it remains to be elucidated. Based on these data, we used NSG mice for the current study to maximize reproducibility and detection sensitivity when comparing the metastatic potential of KTP and WKTP organoids" (page 16).

Reviewer #3 (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.

Reviewer #4 (Remarks to the Author):

The authors satisfactorily addressed most of my concerns. A better characterization of the

polarization state of recruited macrophages in their GEMMs could further improve the understanding of the role of immune cells in liver metastasis in these models.

➔We appreciate Reviewer #4's suggestion to further characterize macrophage polarization. Accordingly, we performed immunohistochemistry for CD206, an M2 macrophage marker, on serial sections alongside F4/80, and found $CD206^+$ M2-like macrophages in the early stage of WKTP cell liver metastatic tumors. We have added these results in the Supplementary Fig. 5, and revised the Results section as "On day 7 after transplantation, numerous small metastatic lesions of WKTP cells were detected in the liver, accompanied by activated fibroblasts and infiltrating $CD206^+$ M2-like macrophages (Fig. 3h, i, and Supplementary Fig. 5)" (page 8). Moreover, we have briefly discussed the role of Wnt signaling in M2 polarization in Discussion as "Consistent with prior reports that Wnt/β-catenin signaling promotes M2 polarization of tumor-associated macrophages (TAMs) (32), we observed $CD206^+$ M2-like macrophages in the liver micrometastatic lesions. These results suggest that Wnt-induced M2 TAMs may facilitate metastatic progression" (page 15).

Reviewer #5 (Remarks to the Author):

Furutani et al have provided the revised manuscript. Overall reviewers comments regarding the consideration of relevant literature, CAF-related language have been corrected. However, the key problems have not been experimentally addressed (except the new CAF in vitro experiments in the Fig. 6B and 7B). Unfortunately, two key points remain unanswered and that is:

1) relevance to human: only very limited data, with reduced statistical significance, have been provided from human samples to indicate the some of the observations might hold true in human samples.

➔We appreciate the Reviewer's emphasis on human relevance. Our study was designed to mechanistically interrogate ligand-dependent Wnt signaling in gastric cancer liver metastasis using genetically controlled mouse models and organoid transplantation systems.

We agree that definitive validation in human tissues is essential. While our current dataset includes only limited human evidence, the convergence of multiple independent, genetically defined models provides robust proof-of-concept for a previously underappreciated mechanism of metastasis.

In recognition of this limitation, we revised the Discussion to state: "Our present study supports a model in which ligand-dependent Wnt signaling and CAF-derived Has2 promote hyaluronan deposition in the tumor stroma to facilitate gastric cancer metastasis. While these results were obtained in genetically controlled mouse and organoid models, prospective

validation in human gastric cancer liver metastasis is warranted, and will be an important focus of our future work" (page 17).

2) take more care about the cell of origin. It is not OK to use cancer cells to express stromal genes even if these are supposed to be secreted and exert their activity outside the cell. One does not know if these proteins may have functions in the cytoplasm. Hence, if HYAL1 is expressed in endothelial cells, then a model must account for that and expressing HYAL1 in cancer cells will not be considered as relevant.

➔ We appreciate the reviewer's insightful comment regarding the importance of considering the cell of origin when assessing the function of stromal genes.

In our study, we ectopically expressed hyaluronidases (Hyal1 and Hyal2) in WKTP cancer cells to experimentally degrade hyaluronan in the tumor microenvironment of liver metastases. This strategy was adopted to functionally assess the role of extracellular hyaluronan in metastatic niche formation. Our data showed that expression of these enzymes significantly suppressed liver metastasis, suggesting a critical role for hyaluronan deposition in the metastatic microenvironment.

We acknowledge the reviewer's concern that expressing stromal genes in cancer cells may potentially introduce intracellular effects unrelated to their physiological function. To address this, we have confirmed that expression of Hyal1/Hyal2 in WKTP cells did not affect their *in vitro* proliferation as well as *in vivo* tumorigenic potential, indicating that their primary effect is likely exerted extracellularly (see Supplementary Fig. 10) (page 13).

That said, we fully agree that evaluating the role of stroma-derived hyaluronan more physiologically—such as by targeting *Has2* in cancer-associated fibroblasts (CAFs)—will provide more definitive insights. Accordingly, we have clarified this point in the revised manuscript as follows: "This hypothesis warrants further investigation, including studies specifically targeting *Has2* in stromal fibroblasts to delineate its functional role in the metastatic process" (page 16).

Next to this, there are several points that need to be addressed to clarify if the data from the manuscript could be reproduced in the future. In particular the authors should:

1) make sure that reviewers now and other scientist later can access the OMICS data. The link that has been provided does not work.

➔At the time we submitted the revision, the DDBJ records were still under hold, which caused the temporary dead link. We have since released the records to public access, and we have verified that all datasets cited in the Data Availability section are now accessible via their accession numbers.

2) the WKTP gastric cancer model, as well as all other models that are not publicly available yet used in the present manuscript, need to be outlined in the "Material Availability" section, where the conditions and accessibility for sharing these models should be clearly stated.

➔According to the Reviewer #5's suggestion, we have added the information of availability of mouse and organoid lines used in this study in Materials Availability section as "WKTP and KTP mouse models, and WKTP, KTP, *Apc*-KTP, and *Hyal*-KTP organoids are available from the corresponding author upon reasonable request and completion of a Material Transfer Agreement" (page 27).

Reviewer #6 (Replacing Reviewer #1, Remarks to the Author):

Furutani and colleagues present a compelling study identifying a novel role for Wnt1-dependent signaling in conjunction with hyaluronan-expressing cancer-associated fibroblasts as a key mediator of gastric cancer liver metastasis. Using diverse methodologies, they demonstrate that cell-intrinsic Wnt signaling alone is insufficient for metastasis induction; instead, synergistic Wnt and TGFβ signaling induces hyaluronan in stromal cells. The study is strengthened by robust genetic control populations and inhibition models. The authors adequately addressed previous reviewer concerns, with manuscript revisions enhancing the overall quality, and I support its publication in Nature Communications.

➔We thank the Reviewer #6 for the positive assessment of our manuscript and support for publication.

Minor concerns are outlined below.

1) Lines 113–114 claim all mutant mice showed significantly increased thickness, but the statistical comparison of WT vs. K is missing in Figure 1c.

➔Thank you for pointing this out. We re-checked the statistics using a one-way ANOVA followed by Tukey's post-hoc test across all genotypes. The comparison between wild-type (WT) and the simple *Kras* mutant (K) was not significant; all other mutant groups showed significantly increased gastric mucosal thickness vs WT. We have updated Fig. 1c to label the WT vs K comparison as n.s., and we revised the text as "All mutant mice except the simple *Kras* mutant group showed significantly increased gastric mucosal thickness compared with wild-type mice (Fig. 1b, c)" (page 5)

2) Supplementary Figure 8b shows a correlation between HAS2 and WNT6, but WNT6 is not mentioned in the text.

➔WNT6 has been reported to act predominantly through the canonical β-catenin pathway. Thus, we have revised the Results as "Moreover, we observed a modest but positive

correlation between *HAS2* and canonical Wnt ligands such as *WNT2*, *WNT2B*, and *WNT6* (the latter has been reported to act predominantly through the canonical Wnt signaling), suggesting a potential role for canonical Wnt signaling in the regulation of *HAS2* expression in human gastric cancer (Supplementary Fig. 8b)" (page 12).

3) Figures 6 and 7 label "Afamin-Wnt3," while the text uses "Afamin-Wnt3a." Relabel figures as "Afamin-Wnt3a" for consistency.

➔As the Reviewer #6 suggested, we have relabeled "Afamin-Wnt3" as "Afamin-Wnt3a" in Figures 6 and 7.

4) The response to Reviewer #1's concern about using immunodeficient vs. immunocompetent mice is well-articulated. The referenced study (Sakai et al., Cancer Res, 2018) should be cited in the paper, and including parts of this explanation in the discussion would strengthen the experimental design and conclusions.

➔We thank the Reviewer #6 for this helpful suggestion. We have added an explanation in of our rationale for using NSG mice rather than syngeneic C57BL/6 mice with citation of our previous study (Sakai et al, Cancer Res, 2018) in Discussion as "In our previous study, spleen transplantation of intestinal tumor-derived AKTP organoids, harboring mutations in *Apc*, *Kras*, *Tgfbr2*, and *Trp53* into syngeneic C57BL/6 (B6) mice yielded liver metastasis in ~40% of recipients, whereas injection into immunodeficient NSG mice resulted in nearly 100% penetrance (37). This discrepancy is likely due to the NK cell-mediated surveillance in B6 mice, although it remains to be elucidated. Based on these results, we used NSG mice for the current study to maximize reproducibility and detection sensitivity when comparing the metastatic potential of KTP and WKTP organoids" (page 16).