

Metastasis of colon cancer requires Dickkopf-2 to generate cancer cells with Paneth cell properties

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eLife Assessment

This **valuable** study proposes that protein secreted by colon cancer cells induces cells with Paneth-like properties that favor colon cancer metastasis. The evidence supporting the conclusions is **solid** but the study would benefit from more direct experiments to test the functional role of Paneth-like cells and to monitor metastasis from colon tumors. The work will be of interest to researchers studying colon cancer metastasis.

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Abstract

Metastasis is the leading cause of cancer-related mortality. Paneth cells provide stem cell niche factors in homeostatic conditions, but the underlying mechanisms of cancer stem cell niche development are unclear. Here we report that Dickkopf-2 (DKK2) is essential for the generation of cancer cells with Paneth cell properties during colon cancer metastasis. Splenic injection of *Dkk2*-knockout (KO) cancer organoids into C57BL/6 mice resulted in a significant reduction of liver metastases. Transcriptome analysis showed reduction of Paneth cell markers such as lysozymes in KO organoids. Single cell RNA sequencing analyses of murine metastasized colon cancer cells and patient samples identified the presence of lysozyme positive cells with Paneth cell properties including enhanced glycolysis. Further analyses of transcriptome and chromatin accessibility suggested Hepatocyte nuclear factor 4-alpha (HNF4A) as a downstream target of DKK2. Chromatin immunoprecipitation followed by sequencing analysis revealed that HNF4A binds to the promoter region of *Sox9*, a well-known transcription factor for Paneth cell differentiation. In the liver metastatic foci, DKK2 knockout

rescued HNF4A protein levels followed by reduction of lysozyme positive cancer cells. Taken together, DKK2-mediated reduction of HNF4A protein promotes the generation of lysozyme positive cancer cells with Paneth cell properties in the metastasized colon cancers.

Introduction

Metastasis is the main cause of cancer-related death (1). Mutations in the *Apc* gene in intestinal epithelial cells cause adenoma formation by dys-regulation of Wnt signaling (2). Accumulating oncogenic mutations in *Kras*, *Tp53* and *Smad4* genes facilitate colorectal carcinogenesis and metastasis (3–5). Recent studies have identified that *Lgr5* positive cells are required for metastasis in the murine models of colorectal cancer (6, 7). *Lgr5* positive cells include stem cells, transit amplifying cells and Paneth cells (8). Ablation of *Lgr5* positive cells in the primary tumor in mice restricted metastatic progression of colon cancer cells (6). Time-lapse live imaging analysis of metastatic cancer cells in the murine primary colon tumors revealed that both *Lgr5* positive and negative cancer cells are able to escape from the primary tumors and circulate in the blood stream (7). Importantly, generation or presence of *Lgr5* positive cells is necessary to form metastases suggesting that cancer stem cells and their niche formation upon metastases seeding is a prerequisite of metastatic tumor growth.

The stem cell niche provides so called ‘stem cell niche factors’ in order to regulate proliferation and differentiation of stem cells (9, 10). In the homeostatic condition, Paneth cells function as stem cell niche cells by providing Wnt3, EGF, Notch ligand, and Dll4 to intestinal stem cells (11). Paneth cell-mediated glucose conversion into lactate promotes proliferation of intestinal stem cells that lack glucose metabolism (12). Stem cell niche factors not only maintain stem cells but also generate new stem cells by dedifferentiation from the progenitors when the original stem cells are damaged by intestinal injury or inflammation (13–15). Regeneration of stem cells by niche factors also occurs in the context of colorectal cancer (6, 16, 17). However, the underlying mechanisms of cancer stem cell niche cell development in the primary and metastatic colon tumors are largely unknown.

In normal mucosa, the balance between Wnt and Notch signaling determines the fate of intestinal stem cells differentiating to secretory or absorptive precursors (18–20). *Atoh1* expression in the secretory precursors directs their differentiation to Paneth or goblet cells (21). *Sox9* expression then induces Paneth cell differentiation (22, 23) in the small intestine. Recent findings have identified the presence of Paneth-like cells in the large intestine (24–26). However, the existence of Paneth or Paneth-like cells in colon cancers remains unknown. Using colon cancer organoids carrying mutations in *Apc*, *Kras* and *Tp53* genes, we showed here that *Dkk2* knockout disrupted Lysozyme (LYZ) positive cell formation in colon cancer organoids as well as metastatic nodules *in vivo*. Single cell RNA sequencing analyses for mouse and human metastatic colon cancer samples have identified the existence of LYZ+ cells exhibiting Paneth cell properties including metabolic support for stem cells (27). Mechanistically, DKK2 protein deficiency recovered protein levels of Hepatocyte Nuclear Factor 4 alpha (HNF4A) in colon cancer cells (28). HNF4A binding on the *Sox9* promoter inhibited the formation of LYZ+ cancer cells exhibiting Paneth cell characteristics. The loss of LYZ+ cells by *Dkk2* knockout rescued mice from the liver metastasis of colorectal cancer induced by cancer organoid transplantation. Our findings suggest that DKK2 promotes LYZ+ cell formation exhibiting Paneth cell properties to develop cancer stem cell niches for outgrowth of metastasized colon cancers.

Results

DKK2 is indispensable for liver metastasis of colorectal cancer

Metastatic potential of *Lgr5*-expressing cells has been reported in colorectal cancer (29). The ablation of *Lgr5*-expressing cells in primary colon tumors inhibited metastasis whereas the growth of primary tumors quickly recovered by dedifferentiation of non-stem cells into *Lgr5*-expressing stem cells (6). Moreover, Fumagalli *et al.* have shown that *Lgr5*-expressing cells are indispensable for the growth of metastases (7). We have recently reported that DKK2 enhanced *Lgr5* expression in colon cancers through activation of c-Src (28). Enhanced activation of c-Src has been reported in metastatic colon cancers as well (30). Based on these observations, we queried whether DKK2 is required for metastasis of colorectal cancer. We developed colon cancer organoids carrying oncogenic mutations in *Apc*, *Kras* and *Tp53* genes (AKP) (28). Splenic injection of the *Dkk2* knockout AKP (KO) organoids into wild type C57BL/6 mice resulted in significantly less liver metastasis and mortality compared to the control (AKP) organoids (Fig. 1A-D). Quantitative gene expression analyses revealed that the levels of *Dkk2* and *Lgr5* were still decreased in KO-derived liver metastasized cells compared to AKP-derived cells (Fig. 1E). *Lgr5* protein expression and c-Src phosphorylation were reduced in KO-derived liver tumors (Fig. 1F and G). The percentage of *Lgr5*-expressing cells with c-Src phosphorylation in KO tumor tissues was decreased about 2-fold compared to AKP tissues (Fig. 1H). These data suggest the necessity of DKK2 in the growth of *Lgr5*-expressing cancer stem cells in liver metastasized nodules originated from the splenic injection of cancer organoids.

DKK2 is required for lysozyme positive cell formation in colon cancer organoids

To understand the underlying mechanisms of the DKK2-mediated increase of *Lgr5*-expressing stem cells in liver metastases, we performed RNA sequencing (RNA-seq) analysis on AKP and *Dkk2* knockout (KO) organoids. KO organoids were generated using the CRISPR technique and DKK2 reconstitution was followed using recombinant mouse DKK2 protein (Fig. 2A). Consistent with our previous report using a colitis-induced tumor model, expression of stem cell marker genes including *Lgr5* was reduced in KO organoids (Fig. S1A) (28). In particular, the expression of Paneth cell marker genes such as *Lyz1*, *Lyz2*, and alpha defensins were significantly reduced in KO organoids (Fig. 2B and S1B). Paneth cells are derived from *Lgr5*-expressing stem cells by asymmetric division and maintain their *Lgr5* expression (15). These cells produce stem cell niche factors such as Wnt3A in order to facilitate stem cell proliferation and differentiation into other epithelial lineage cells (11). Paneth cells are also crucial for the glycolysis of intestinal stem cells that lack of glucose metabolism (12). Our bulk RNA-seq data showed that Paneth cell-derived Wnt3a as well as *Fgfr3*, a marker of Paneth cell-induced expansion of stem cells were reduced by *Dkk2* knockout, indicating that the formation of stem cell niche might be disrupted in KO organoids (Fig. S1C) (31). Indeed, formation of Lysozyme positive (LYZ+) cells was disrupted by *Dkk2* knockout during organoid culture (Fig. 2C). LYZ+ cells were detected in AKP organoids from day 2 after plating a single or multiple cells of enzyme-digested organoids whereas those cells were less frequently observed in KO organoids. It correlates to the expression pattern of *Lgr5* in our previous report (28). A significant increase of *Lgr5* expression was observed between day 2 and day 4 and peaked at day 4 to day 6 in AKP organoid culture.

Lgr5 expression was reduced about 3-fold in KO organoids compared to AKP organoids at day 8 (28). Likewise, the markers of Paneth cells, *Lyz1* and *Lyz2* were reduced in KO organoids at day 8 and recombinant mouse DKK2 protein treatment into KO organoids partially rescued it (Fig.

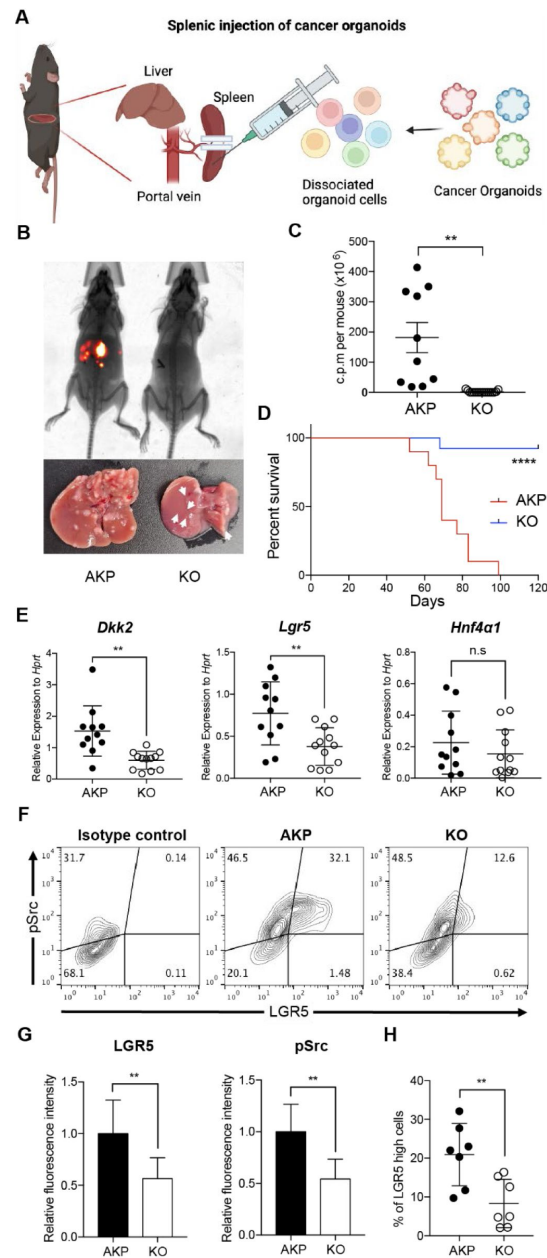


Figure 1.

DKK2 is required for Lgr5 positive stem cells-driven liver metastasis of colorectal cancer.

(A) Isolated cells from AKP tumor organoids expressing the *tdTomato* reporter gene were dissociated and injected into the spleen of wild type C57BL/6 mice. 8 weeks after the injection, metastatic tumor growth was measured by *in vivo* imaging analysis. (B) Representative pictures of *in vivo* imaging analysis. Ctrl: AKP control organoids transduced with scrambled guide RNA, KO: *Dkk2* knockout AKP organoids. (C-D) Statistic analysis of liver metastasis (C) and survival (D). Ctrl ($n=10$), KO ($n=15$), n represents the number of mice. c.p.m. in (C): count per minutes (E) Quantitative gene expression analysis of *Dkk2*, *Lgr5* and *Hnf4a1* in liver metastasized colon cancer cells. Ctrl ($n=11$), KO ($n=12$), n represents the number of isolated cancer nodules with 5 mice per group. (F) Representative flow cytometry analysis data of c-Src phosphorylation (pSrc) and *Lgr5* expression in metastasized colon cancer cells. (G) Statistic analysis of *Lgr5* expression and pSrc in (E). The average of mean fluorescence intensity in control samples was set as 1 and relative fluorescence intensity was calculated. (H) Statistic analysis of the percentile of Lgr5 high (Lgr5 and pSrc double positive) cells in metastasized colon cancer in (F). Each symbol represents an individually isolated cancer nodule. n.s = not significant, ** $P<0.01$, **** $P<0.0001$; two-tailed Welch's t-test (C, E, G, H). Error bars indicate mean $\pm$ s.d. Log-rank test (D). Results are representative of three independent experiments.

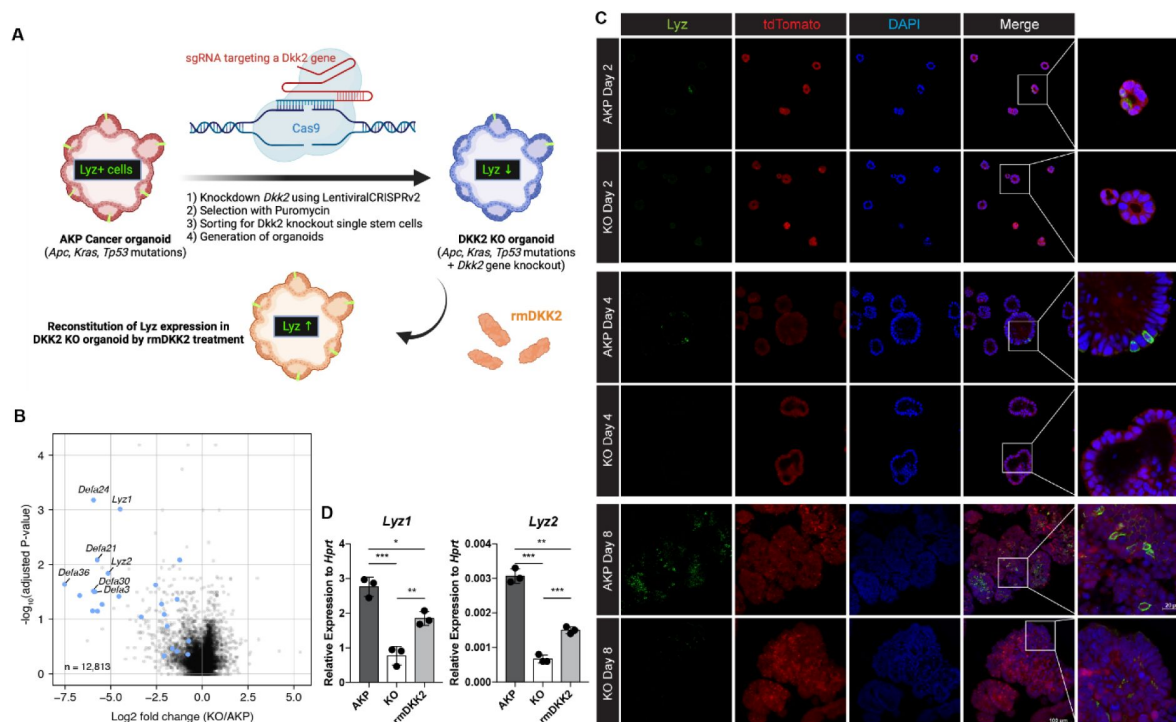


Figure 2.

DKK2 is indispensable for the generation of cancer cells with Paneth cell properties in colon cancer organoids.

(A) A schematic diagram of the generation of KO organoids using CRISPR technique and the reconstitution of DKK2 in organoids by recombinant mouse DKK2 protein (rmDKK2) treatment. Lysozyme (LYZ) expression is highlighted for the followings. **(B)** A volcano plot of RNA-seq analysis comparing KO versus AKP organoids. Paneth cell marker genes are highlighted as blue circles (AKP=3 and KO=5 biological replicates were analyzed). **(C)** Confocal microscopy analysis of LYZ positive cells in AKP or KO organoids in a time-dependent manner using anti-Lysozyme (LYZ) antibody. **(D)** Quantitative real time PCR analyses of *Lyz1* and *Lyz2* in 8 days cultured colon cancer organoids. KO organoids were cultured in the presence of 1 μ g/ml of recombinant mouse DKK2 protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed Welch's t-test. Error bars indicate mean $\pm$ s.d. Results are representative of three biological replicates.

2D [2D](#)). These data indicate that DKK2 is necessary for LYZ⁺ cell formation in colon cancer organoids that might be required for the cancer stem cell niche formation.

LYZ⁺ cancer cells exhibit Paneth cell properties in both mouse and human systems

To investigate the necessity of DKK2 in the formation of LYZ⁺ cancer cells during colon cancer metastasis, we employed the murine *in vivo* model of liver metastasis shown in **Figure 1** [1](#). In order to analyze transcriptome changes by *Dkk2* knockout in each cell type of metastasized tumors, we performed single-cell RNA sequencing (scRNA-seq) analysis on liver metastasized AKP and KO tumor cells. The Louvain method revealed 4 clusters in our dataset. A cell type was assigned to each cluster on the basis of gene expression patterns and visualized in a Uniform manifold approximation and projection (UMAP) representation (**Fig. 3A** [2](#)). Epithelial tumor cells were sub-clustered as 7 clusters and the cluster 6 has been identified as cancer cells with Paneth cell properties based on the expression of Paneth marker genes using the Panglao database (**Fig. 3A** [2](#), S2A-D). Total of 17 cells in cluster 6 were further defined as cancer cells with Paneth cell properties (Paneth⁺) or goblet cell properties (Goblet⁺) using the relevant marker genes expression, *Lyz1*, *Muc2* and *Atoh1* (**Fig. S2E** and **S2F**). The calculated ratio between Paneth⁺ cells and Goblet⁺ cells in KO metastasized tumors was 0.43, reduced as half compared to the ratio in the AKP control, 1 (**Fig. S2F**). This suggests a reduction of LYZ⁺ cells in metastasized colon tumors by knockout a *Dkk2* gene.

To analyze the consequence of the loss of LYZ⁺ cells by *Dkk2* knockout in cancer stem cell niche formation, we enriched *Lgr5* positive cells that consist of stem cells, stem cell niche cells and transit amplifying cells in the homeostatic condition (**Fig. S3A** and **S3B**) (**8** [3](#)). Elevated expression of stem cell marker genes represents cancer stemness in *Lgr5* positive cells compared to *Lgr5* negative cells (**Fig. S3C**). The UMAP plot of *Lgr5* positive cells displayed 2 clusters (**Fig. S3D**). Interestingly, expression of Noggin (*Nog*), one of the stem cell niche factors was detected mostly in cluster 1 where only 1 cell was counted in KO (**Fig. S3E-S3G**). Noggin is an inhibitor of BMP signaling, which restricts stemness of *Lgr5* positive stem cells in the gut (**32** [4](#), **33** [5](#)). Following the reduction of *Nog*, *Bmp4* expression was reversed in KO tumor epithelial cells in the scRNA-seq data (**Fig. S3H**). This has been confirmed by quantitative gene expression analysis of *Bmp4* in KO organoids (**Fig. S3I**). The correlative expression between *Dkk2* and the stem cell marker gene, *Lgr5* has been shown in the scRNA-seq data (**Fig. 3B** [2](#)). Furthermore, pathways enrichment analysis of the scRNA-seq data using IPA suggested that the glycolysis I pathway was significantly inhibited in KO *Lgr5* positive tumor epithelial cells (**Fig. 3C** [2](#)). Expression of glycolysis-related genes such as *Aldoa*, *Aldoc* and *Fbp2* was reduced by *Dkk2* knockout in *Lgr5* positive cells (**Fig. 3D** [2](#)) (**34** [6](#)). Previous studies have defined that Paneth cells participate in the regulation of energy metabolism in intestinal stem cells (**12** [7](#), **35** [8](#)). Since stem cells are not able to generate lactate, Paneth cells instead process glucose in order to provide lactate to stem cells (**12** [7](#)). Therefore, reduction of the glycolysis I pathway in the absence of DKK2 indicates the impaired stem cell niche formation by the loss of LYZ⁺ cells carrying Paneth cell properties.

Paneth cells constitute the stem cell niche in the small intestine (**21** [9](#)). Recent studies have identified the presence of Paneth-like cells in the colon (**25** [10](#), **26** [11](#)). However, the presence of Paneth cells or Paneth-like cells within the tumor microenvironment is elusive. To discern the existence of cancer cells exhibiting Paneth cell properties in humans, we conducted an analysis of scRNA-seq data obtained from colorectal cancer patients (**36** [12](#)). Our analysis focused on CD45-EpCAM⁺ epithelial cells, excluding hematopoietic cells. UMAP plotted 31 clusters of single cells derived from normal tissue, primary tumor and metastasis samples (**Fig. 4A and B** [2](#)). Employing lysozyme expression and Paneth cell module scores (Panglao database) as criteria, we have identified LYZ⁺ cancer cells harboring Paneth cell properties across 5 clusters (**Fig. 4C-H** [2](#), **Fig. S4** and **S5**). LYZ⁺ cancer cell population is distinct in metastasis samples (**Fig. 4E** [2](#) and **4F** [2](#)). LYZ

expression over 2.0 (3rd Quantile, round 1) and Paneth module scores over 0.2 (3rd Quantile, round 2 to avoid getting 0) single cells were identified as LYZ+ cancer cells harboring Paneth cell properties, 1%, 13% and 24% in normal, primary tumor and metastasis samples, respectively.

This delineates the distribution of LYZ+ cancer cells with Paneth cell characteristics in different stages of colorectal cancer progression.

To unravel the functional significance of LYZ+ cancer cells, we performed gene set enrichment analysis for the scRNA-seq data obtained from colon cancer patients. In correlation with our murine scRNA-seq data IPA results, glycolysis emerged as the 2nd-ranked enriched pathway in LYZ+ cancer cells compared to LYZ- cancer cells (**Fig. 5A** [↗](#)). Notably, key players in glycolysis such as *ALDOB* and *KDEL3* genes are increased in LYZ+ cancer cells (**Fig. 5B** [↗](#)). Also, the hallmark of protein secretion was the top-ranked enriched pathway, which is essential for Paneth cell activity including secretion of anti-microbial peptides (**Fig. 5A** [↗](#)). These results suggest the existence of a distinct population of LYZ+ colon cancer cells endowed with Paneth cell properties. Collectively, DKK2 is required for the generation of LYZ+ cells to form the cancer stem cell niche, a prerequisite for the outgrowth of metastasized colorectal cancer cells.

HNF4A mediates the formation of Lysozyme positive colon cancer cells by DKK2

Ingenuity pathway analysis (IPA) of the human scRNA-seq data from primary tumor and metastasis samples suggested hepatocyte nuclear factor 4 alpha (HNF4A) as an upstream regulator of LYZ+ cancer cells compared to LYZ- cancer cells (**Fig. 5C** [↗](#)). In line with this, IPA of the bulk RNA-seq data using mouse colon cancer organoids suggested that HNF4A is activated in KO organoids (**Fig. 6A** [↗](#)). This is consistent with our previous report that DKK2 enhanced *Lgr5* expression in colitis-induced cancer cells via HNF4a1, an isoform of HNF4A ([28](#) [↗](#)). Significant enrichment of the HNF4A binding motif was also detected in the open chromatin regions of KO organoids compared to AKP controls determined by assay for transposase-associated chromatin using sequencing (ATAC-seq) (**Fig. 6B** [↗](#)). In comparison with normal colonic organoids, the HNF4A motif was listed as the most reduced motif in AKP indicating significant reduction of HNF4A-regulated transcription whereas it might be rescued by *Dkk2* knockout (**Fig. 6C** [↗](#)). Notably, HNF4A was the only molecule suggested by all of these multi-omics data analyses in both mouse and human. These results suggest HNF4A as a key transcription factor in downstream signaling of DKK2 in colon cancer cells.

HNF4A is necessary for maturation of fetal intestine and barrier functions of intestinal epithelium in the homeostatic condition ([37](#) [↗](#), [38](#) [↗](#)). Two *Hnf4a* isoforms—*Hnf4a1* and *Hnf4a7*—are involved in intestinal homeostasis and regeneration ([39](#) [↗](#)). Intestinal inflammation induces expression of the HNF4a1 isoform in the crypt epithelial cells to regenerate the epithelium whereas loss of the HNF4a1 isoform occurs in colitis-induced tumor cells ([39](#) [↗](#)). Loss of the HNF4a1 isoform was also observed in AKP organoids while *Dkk2* knockout restored the presence of HNF4a1 protein in the nucleus of AKP organoid cells ([28](#) [↗](#)). This allowed us to investigate the binding regions of HNF4a1 in colon cancer cells using KO organoids. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis using the ChIP-seq grade anti-HNF4A antibody revealed that HNF4A binds to the promoter region of *Sox9* in KO organoid cells (**Fig. 6D** [↗](#)). *Sox9* is a transcription factor required for the secretory lineage of epithelial cell differentiation including Paneth cells ([23](#) [↗](#)). Quantitative gene expression analysis showed that *Dkk2* knockout reduced *Sox9* expression in AKP organoids, which was rescued by recombinant DKK2 protein treatment (**Fig. 6E** [↗](#)). Reduced *Sox9* expression in KO organoids was also reversed by HNF4a1 knockdown (**Fig. 6F** [↗](#)). These data indicate that DKK2-driven loss of HNF4a1 protein leads to *Sox9* expression followed by formation of LYZ+ colon cancer cells. Confocal microscopy analysis confirmed that HNF4a1 knockdown rescued the presence of LYZ+ cancer cells in KO

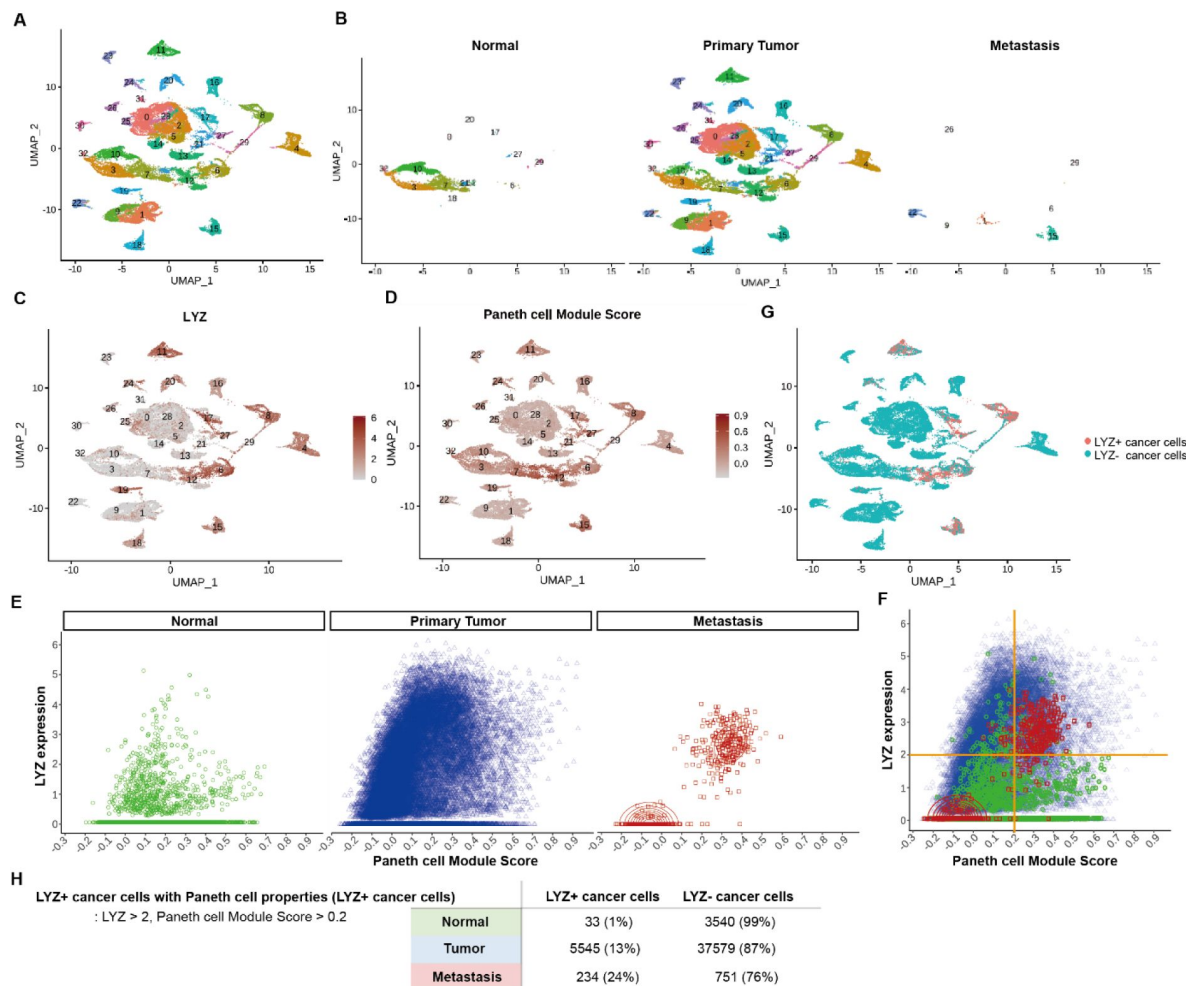


Figure 4.

Identification of colon cancer cells harboring Paneth cell properties in humans.

Published colorectal cancer patient scRNA-seq data (REF) was analyzed to identify the presence of cancer cells with Paneth cell properties. **(A)** The UMAP plot of total 31 clusters. **(B)** Normal cells, primary tumor cells and liver metastasized cells (Metastasis) are shown in the UMAP plot clusters. **(C-D)** Expression levels of lysozyme (LYZ) and Paneth cell module scores are displayed in the UMAP plot. **(E)** Based on the analysis in (C) and (D), cancer cells harboring Paneth cell properties are indicated as red dots (LYZ+ cancer cells). **(F)** Lysozyme expression and Paneth cell module scores of single cells in Normal, Primary Tumor and Metastasis samples are presented by dot plots. **(G)** Dot plots in (F) are overlaid. **(H)** The percentages of cancer cells with Paneth cell properties (LYZ+ cancer cells) are shown.

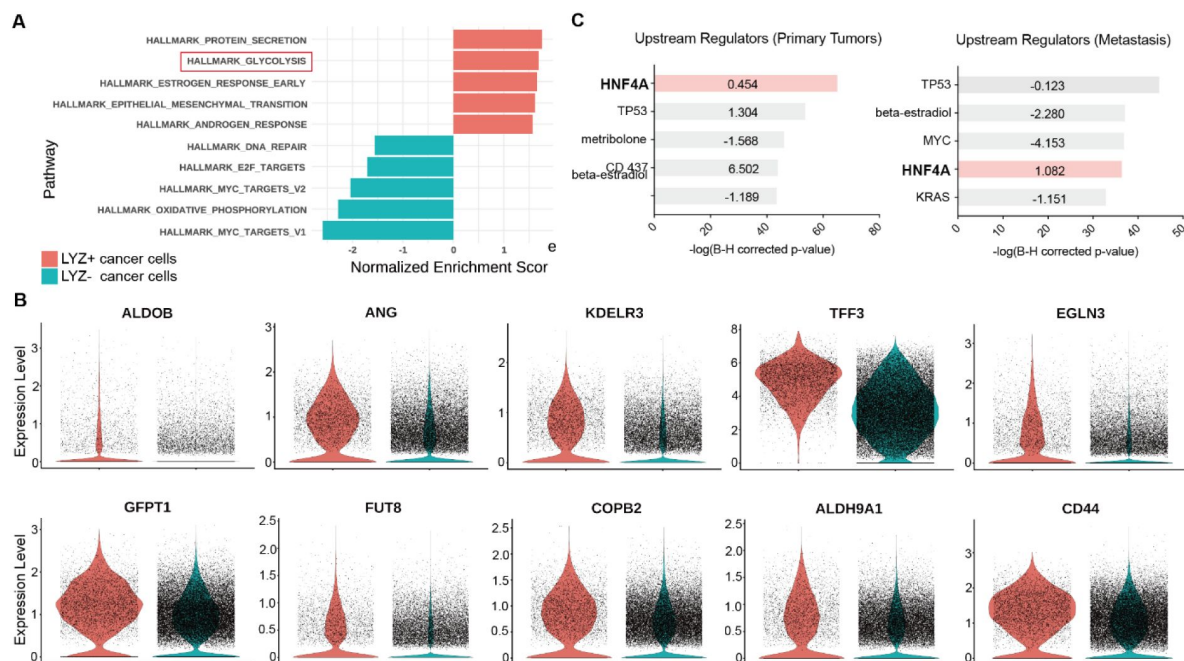


Figure 5.

Colon cancer cells with Paneth cell properties contribute to glycolysis.

Colorectal cancer patient scRNA-seq data were further analyzed by Gene set enrichment analysis (GSEA) and Ingenuity Pathway Analysis (IPA) (A) GSEA of cancer cells with Paneth cell properties (LYZ+ cancer cells) compared to all other cancer cells (LYZ-cancer cells), shown in Fig.4 (G). (B) Representative gene expressions in the hallmark of glycolysis pathway are shown. (C) Upstream regulators suggested by IPA in Primary Tumor and Metastasis are presented. Activation z-scores are indicated in the bar. IPA predicted activation or inhibition of the upstream regulators are colored as red and cyan, respectively.

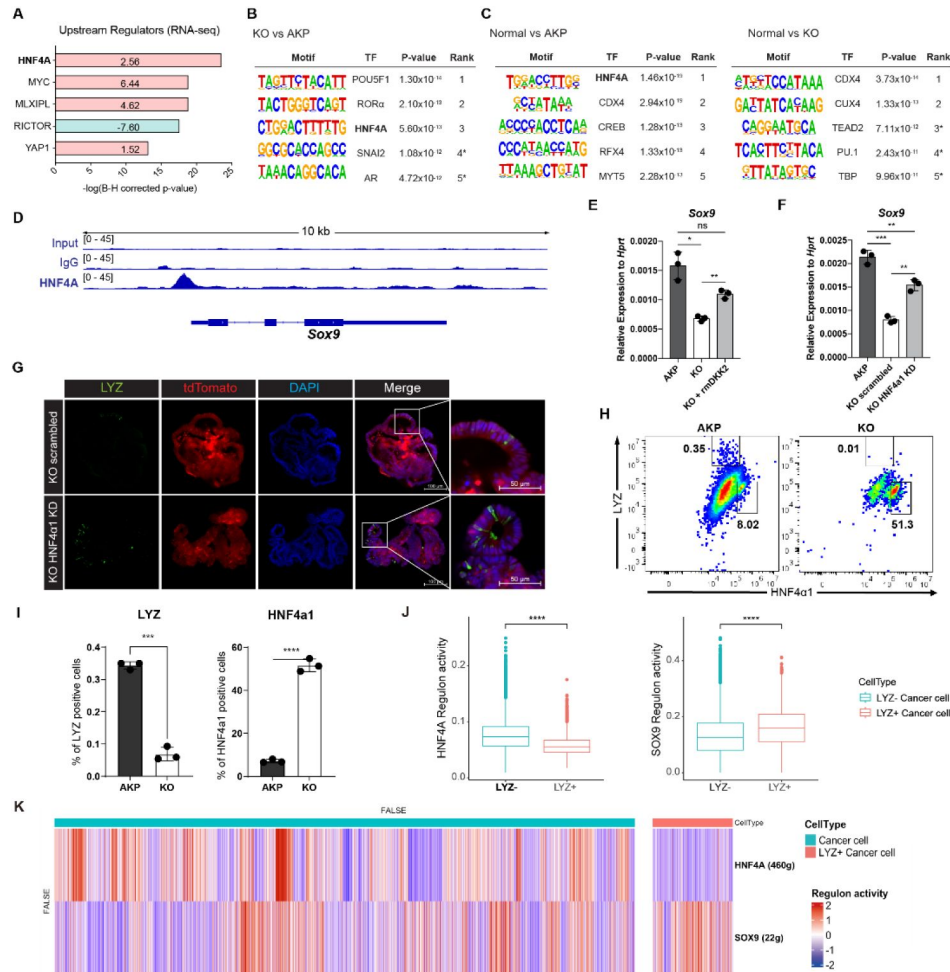


Figure 6.

DKK2-driven reduction of HNF4a1 protein in murine colon cancer organoids promotes cancer cells with Paneth cell properties.

(A) Ingenuity pathway analysis (IPA)-suggested upstream regulators of the RNA-seq data shown in Figure 2A. Z-scores are presented in the bar. IPA predicted activation or inhibition of the upstream regulators are colored as red and cyan, respectively. (B-C) List of top 5 transcriptional factors (TF) in the motif enrichment analysis of ATAC-seq data comparing normal colonic organoids, AKP and KO cancer organoids. * indicates possible false positive. (D) Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of KO organoids using an anti-HNF4A antibody. (E) Quantitative expression of Sox9 in colon cancer organoids in the presence or absence of DKK2 (rmDKK2: recombinant mouse DKK2 protein added in organoid culture). (F) Analysis of Sox9 expression after knockdown HNF4a1 in KO colon cancer organoids (KO HNF4a1 KD). ns = not significant, *P<0.05, **P<0.01, ***P<0.001; two-tailed Welch's t-test. Error bars indicate mean ± s.d. (G) Representative images of confocal microscopy analysis of Lyz-stained cancer cells with Paneth cell properties in DKK2 KO HNF4a1 KD organoids. (H-I) Control AKP or KO colon cancer organoids were transplanted via splenic vein as described in Figure 1. 3 weeks after transplantation, mice were sacrificed to analyze metastatic tumor growth in liver. Quantification of cancer cells with Paneth cell properties in metastasized tumor nodules by flow cytometry for Lysozyme (LYZ) and HNF4a1. Tumor cells were initially gated by the tdTomato reporter expression. Representative images of flow cytometry are shown (H). Statistic analyses of the percentiles of LYZ positive cells (% of upper left) and HNF4a1 positive cells (% of lower right) in tumor nodules (I). **P<0.01, ****P<0.0001; two-tailed Welch's t-test. Error bars indicate mean ± s.d. 3 mice were tested per group. Data are representative of two independent experiments. (J-K) Reduced HNF4A regulation activity with enhanced SOX9 regulation activity in LYZ+ cancer cells in human colorectal cancer scRNA-seq data. Box plots represent the regulon activity of HNF4A and SOX9 in LYZ+ cancer cells. ****P<0.0001; Wilcoxon signed-rank test (J). Z-scaled regulon activities of HNF4A and SOX9 in human colon cancer cells is displayed by heatmap (K).

organoids (**Fig. 6G** [↗](#)). In order to quantify LYZ⁺ cancer cells in the mouse model of liver metastasis, tdTomato positive cells in the liver were identified as the implanted organoid-derived cancer cells then, LYZ⁺ cells were calculated (**Fig. 6H** [↗](#)). The percentile of LYZ⁺ cells in total cancer cells was decreased about 3-fold in *Dkk2* knockout metastasized cells (KO) compared to the AKP control cells in liver (**Fig. 6I** [↗](#)). Simultaneously, HNF4α1 positive cells were tripled to about 60 percent in KO (**Fig. 6H** [↗](#) and **6I** [↗](#)). These findings suggest that DKK2 is indispensable for the generation of LYZ⁺ cancer cells in liver metastasized nodules by reducing protein levels of HNF4α1. Further analyses for human colorectal cancer scRNA-seq data revealed that HNF4A regulation activity is reduced in LYZ⁺ human colon cancer cells compared to the LYZ⁻ cancer cells while SOX9 regulation activity is significantly increased (**Fig. 6J-6K** [↗](#) and S5) suggesting the key roles of HNF4A and SOX9 in LYZ⁺ cancer cell formation in human. Taken together, DKK2- driven loss of HNF4α1 protein enhances Sox9 expression in colon cancer cells to generate LYZ⁺ cells with Paneth cell properties.

Discussion

We have shown that DKK2 is indispensable for metastatic tumor growth of colorectal cancer in the murine model developed by transplantation of colon cancer organoids carrying mutation in the *Apc*, *Kras* and *Tp53* genes. Bulk RNA-seq analysis of DKK2-deficient colon cancer organoids showed reduction of the marker genes of Paneth cells, which play a role in the stem cell niche development. Single cell RNA-seq analyses for mouse and human metastatic colon cancers have identified the existence of LYZ⁺ cancer cells harboring Paneth cell properties, in particular, glycolysis for stem cells. In the absence of DKK2, both pathway enrichment analysis of the RNA-seq and ATAC-seq data suggested that HNF4A is activated. This is consistent with our previous report in the murine model of colitis-induced tumorigenesis ([28](#) [↗](#)). ChIP-seq analysis using an anti-HNF4A antibody revealed that HNF4A directly binds to the promoter region of *Sox9*, a transcriptional factor required for epithelial differentiation into Paneth cells ([22](#) [↗](#), [23](#) [↗](#)). Notably, DKK2 induces the loss of HNF4α1 isoform in colon cancer cells followed by elevation of *Sox9* expression lead to the formation of LYZ⁺ cancer cells exhibiting Paneth cell properties. DKK2-deficient colon cancer cells possessed high levels of HNF4α1 protein and failed to generate LYZ⁺ cancer cells *in vitro* and *in vivo*. DKK2 deficiency resulted in reduced glycolysis in mouse liver metastasized colon cancer cells. These results demonstrate the significant reduction of liver metastasis in DKK2 knockout (KO) colon cancer cells infused mice.

Fumagalli et al. have recently shown that the generation of *Lgr5* positive cells from the metastasized *Lgr5* negative cancer cells is necessary for their outgrowth using *in vivo* time-lapse imaging and organoid culture of metastatic cancer cells that escaped from the primary colon cancer tissues ([7](#) [↗](#)). This finding implies the necessity of both cancer stem cells and their niche for metastatic outgrowth. Indeed, Paneth cell-derived stem cell niche factors such as Wnt3, EGF and Dll4 are essential for the maintenance of stem cells ([11](#) [↗](#)). Dietary induced mouse sporadic intestinal cancer showed elevation of Paneth cell marker genes in the intestinal epithelium ([40](#) [↗](#)). IDO1⁺ Paneth cells contribute to the immune evasion of colon cancer ([41](#) [↗](#)). Depletion of Paneth cells or Paneth cell-derived Wnt3 in *Apc*^{Min} mice impaired intestinal adenoma formation ([42](#) [↗](#)). The plasticity of Paneth cells allows dedifferentiation of Paneth cells into intestinal stem cells ([15](#) [↗](#), [43](#) [↗](#)). These reports indicate the necessity of the Paneth cells during the development of colon cancer.

However, the existence of Paneth cells in large intestine or tumor tissues remains unclear. In 2016, Reg4⁺ secretory cells have been suggested as Paneth-like cells in the colon ([24](#) [↗](#)). Single cell transcriptome analysis showed the presence of Paneth-like cells in human colon ([25](#) [↗](#)). Recent study further supported the presence of Paneth-like cells within the colonic stem cell niche during bifidobacterium-induced intestinal stem cell regeneration ([26](#) [↗](#)). Our study suggests the presence

of LYZ⁺ cells exhibiting Paneth cell properties in the context of colon cancer and metastasis. This extends our knowledge of colorectal cancer progression that DKK2 is required for the generation of LYZ⁺ cells forming cancer stem cell niches.

Further investigation will be needed to characterize LYZ⁺ cancer cells with Paneth cell properties observed in colon cancers carrying mutations in *Apc*, *Kras* and *Tp53* genes compared to normal Paneth cells in order to identify their roles in cancer stem cell niche development.

Recent studies have reported stem cell niche remodeling in the primary tumor in the presence of oncogenic mutations. Cancer stem cells outcompete their neighboring normal stem cells during intestinal tumorigenesis, which is known as clonal fixation (44). *Apc*-mutant cancer cells secrete several Wnt antagonists such as NOTUM to inhibit proliferation of normal intestinal stem cells and facilitate their differentiation (45). Other oncogenic mutations in *Kras* or *Pi3kca* genes lead to paracrine secretion of BMP ligands, remodeling the stem cell niche that is detrimental for maintaining normal stem cells (46). These changes are beneficial for the outgrowth of cancer stem cells in the crypt where the stem cell niche had been developed prior to tumorigenesis. In the context of metastasis, the formation of stem cell niches is required upon metastases seeding in the liver. We have shown that DKK2 is required for the formation of LYZ⁺ cancer cells carrying Paneth cell properties. DKK2 expression is induced by *Apc* knockout and further increased by additional oncogenic mutations in *Kras* and *Tp53* genes (28). Characterization of DKK2-induced LYZ⁺ cancer cells will provide better understanding of the biology of cancer stem cell niches, particularly in liver metastases of colorectal cancer.

DKK2 expression in colon cancer promotes LYZ⁺ cancer cell generation through the regulation of HNF4α1, which suppresses expression of Sox9, the transcription factor for Paneth cell differentiation. It has been recently reported that HNF4α2 isoform acts as an upstream regulator of Wnt3 and Paneth cell differentiation implying the roles of HNF4A for Paneth cell differentiation in the homeostatic condition (47). Using *Villin^{Cre}-Apc^{flxed}* mice, Suzuki et al. have confirmed that HNF4A proteins are completely absent in cancer cells with high β-catenin activity along with increased Sox9 expression (48). Sox9 regulates cell proliferation and is required for Paneth cell differentiation (22, 23). Bi-allelic inactivation of the *Apc* gene induces Sox9 expression in colon cancer (49). Likewise, *Apc* inactivation induces *Dkk2* expression (28). Here we showed that DKK2-mediated HNF4α1 protein degradation enhanced Sox9 expression in colon cancer (Fig. 7). Notably, *Apc* mutation-driven Sox9 expression blocks intestinal differentiation and activates stem cell-like program (49). This is consistent with our previous report that DKK2 enhances *Lgr5* expression in colon cancer (28). In sum, our findings suggest that a Wnt ligand, DKK2 is an upstream regulator of SOX9 expression for enhanced stem cell activity via the formation of LYZ⁺ cells with Paneth cell characteristics in colorectal cancers.

In this study, we have shown that DKK2 promotes liver metastasis of colon cancer. DKK2 is required for the formation of LYZ⁺ colon cancer cells exhibiting Paneth cell properties such as glycolysis for stem cells. Glycolysis in *Lgr5* expressing cancer cells was reduced along with the loss of LYZ⁺ cells by *Dkk2* knockout. As a result, metastatic growth of colon cancer cells was significantly inhibited *in vivo*. These findings suggest the necessity of DKK2 in the development of cancer stem cell niches for developing metastases. Mechanistically, DKK2-mediated degradation of HNF4α1 protein elevated Sox9 expression followed by LYZ⁺ cell formation. Given that DKK2 expression is intestinal tumor specific, inhibition of DKK2 may have promising therapeutic outcomes in the treatment of metastatic colon cancer.

Materials and Methods

In the absence of DKK2

In the presence of DKK2

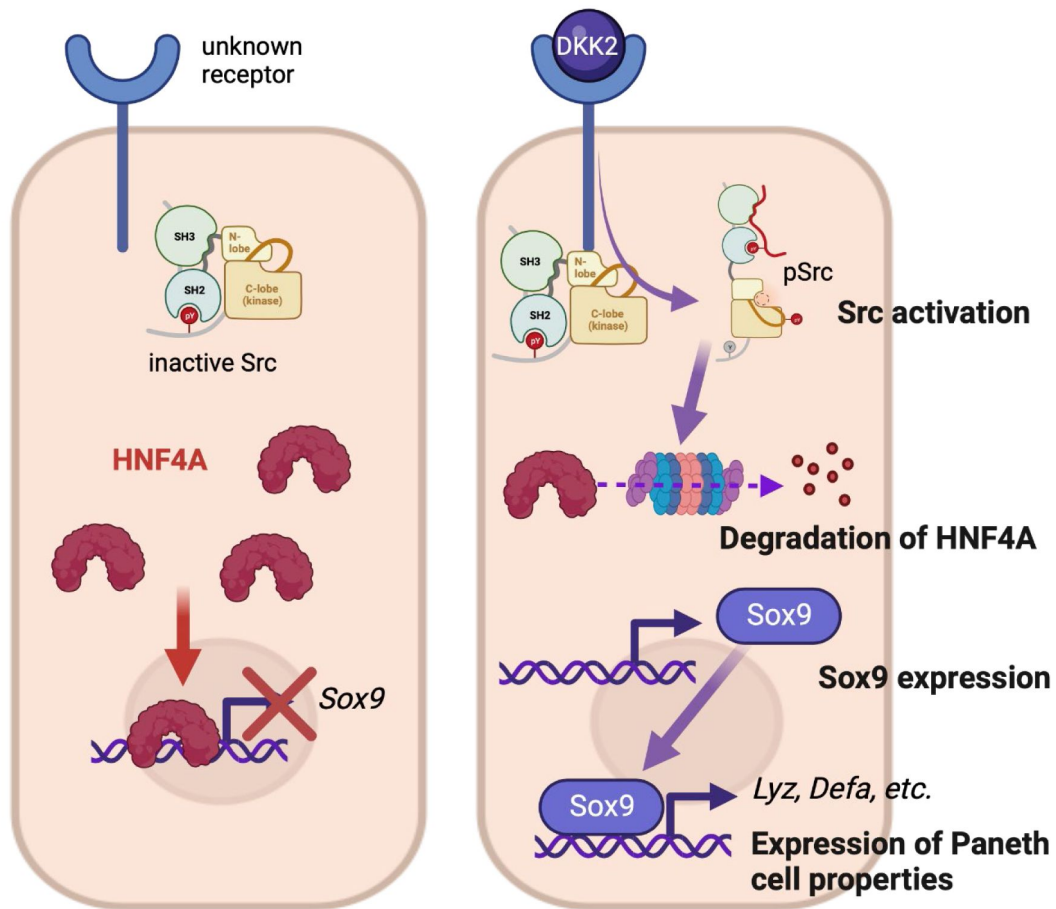


Figure 7.

The suggested mechanism of DKK2 in the formation of colon cancer cells with Paneth cell properties.

In the absence of DKK2, Sox9 expression is inhibited by HNF4A. Our previous report has shown that DKK2 activates Src followed by degradation of HNF4A protein (28). HNF4A deficiency leads to Sox9 expression in colon cancer cells that induces Paneth cell properties including the expression of lysozymes and defensins. Formation of cancer cells with Paneth cell properties by DKK2 contributes the outgrowth of metastasized colon cancer cells in the liver.

Splenic injection

Generation of AKP, *Dkk2* knockout AKP, *HNF4a1* knockdown and *Dkk2* knockout AKP organoids and their culture protocols have been published in the previous study (28). We used the previously reported splenic injection methodology to develop organoid-derived liver metastasis in mice (50). Cultured organoids were prepared as single cells following 10-30 min of digestion in TrypLE solution (Thermo, 12605010) at 37°C, followed by centrifugation with advanced DMEM-F12 (Thermo, 12634010). The isolated single cells were re-suspended with PBS as 300,000 cells per 100 µl of PBS. Surgical instruments were sterilized by steam autoclave (250 ° F., 15 p.s.i. for 30 minutes). 8-12 weeks old C57BL/6 mice were anesthetized with Ketamine (100 mg/kg) plus Xylazine (10 mg/kg) via intraperitoneal injection. Puralube Vet ointment was applied to the eyes to prevent drying while surgery. Spleen was divided by two Horizon medium size ligating clips (Teleflex, 002200) in the center of the spleen and one hemi-spleen was returned into the peritoneum. Using a 26-½ Gauge needle, 100-200 µl volume of cells was injected underneath of the splenic capsule over the course of 30 s. A small Horizon clip (Teleflex, 001200) was applied to ligate the pancreas and splenic vessels and the injected hemi-spleen was removed. The incision was closed with a 5-0 running stitch using Vicryl (Ethicon, J493G) and 2-3 skin clips. Meloxicam (0.3 mg/kg) was injected subcutaneously to alleviate postsurgical pain at 24-72 h post-surgery.

In vivo imaging

Noninvasive *in vivo* imaging was performed using a Microfocus X-ray imaging source (Kodak). Mice were anesthetized by isoflurane inhalation and their abdominal hair was shaved. Tandem repeat Tomato (tdTomato) fluorescence in liver metastatic tumors was recorded for 30 sec. tdTomato signals were statistically analyzed using the Bruker image analysis system. X-ray image was captured by 10 sec exposure and merged with the tdTomato image.

Data availability

All data are included in the article and SI Appendix. All the sequencing data reported in this study have been deposited in the Gene Expression Omnibus as follows; Bulk RNA-seq: GSE157531, ATAC-seq: GSE157529, Chip-seq: GSE277510, scRNA-seq: GSE157645.

Acknowledgements

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Author contributions

J.H.S. and A.L.M.B. designed research; J.H.S., J.L., J.J., R.K.D, S.E.M., and J.Y.H. performed research; J.H.S., J.P., J.L., J.J., R.K.D., S.E.M., J.Y.H., and A.L.M.B. contributed new reagents/analytic tools; J.H.S., J.P., J.J., J.L., J.C., J.W. and A.L.M.B. analyzed data; J.H.S., J.P., J.K., S.P., J.C. performed the revision experiments; and J.H.S., J.J., J.L., J.C. and A.L.M.B. wrote the paper.

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Conflict of interest

The authors declare no competing interests.

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Reviewer #1 (Public review):

Summary:

The authors addressed the influence of DKK2 on colorectal cancer (CRC) metastasis to the liver using an orthotopic model transferring AKP-mutant organoids into the spleens of wild-type animals. They found that DKK2 expression in tumor cells led to enhanced liver metastasis and poor survival in mice. Mechanistically, they associate Dkk2-deficiency in donor AKP tumor organoids with reduced Paneth-like cell properties, particularly Lz1 and Lyz2, and defects in glycolysis. Quantitative gene expression analysis showed no significant changes in Hnf4a1 expression upon Dkk2 deletion. Ingenuity Pathway Analysis of RNA-Seq data and ATAC-seq data point to a Hnf4a1 motif as a potential target. They also show that HNF4a binds to the promoter region of Sox9, which leads to LYZ expression and upregulation of Paneth-like properties. By analyzing available scRNA data from human CRC data, the authors found higher expression of LYZ in metastatic and primary tumor samples compared to normal colonic tissue; reinforcing their proposed link, HNF4a was highly expressed in LYZ+ cancer cells compared to LYZ- cancer cells.

Strengths:

Overall, this study contributes a novel mechanistic pathway that may be related to metastatic progression in CRC.

Weaknesses:

The main concerns are related to incremental gains, missing in vivo support for several of their conclusions in murine models, and missing human data analyses.

Main comments

Novelty:

The authors previously described the role of DKK2 in primary CRC, correlating increased DKK2 levels to higher Src phosphorylation and HNF4a1 degradation, which in turn enhances LGR5 expression and "stemness" of cancer cells, resulting in tumor progression (PMID: 33997693). A role for DKK2 in metastasis has also been previously described (sarcoma, PMID: 23204234)

Mouse data:

(a) The authors analyzed liver mets, but the main differences between AKT and AKP/Dkk2 KO organoids could arise during the initial tumor cell egress from the intestinal tissue (which cannot be addressed in their splenic injection model), or during pre-liver stages, such as endothelial attachment. While the analysis of liver mets is interesting, given that Paneth cells play a role in the intestinal stem cell niche, it is questionable whether a study that does not involve the intestine can appropriately address this pathway in CRC metastasis.

(b) The overall number of Paneth cells found in the scRNA-seq analysis of liver mets was low (17 cells, Fig.3), and assuming that these cells are driving the differences seems somewhat far-fetched.

(c) Fig. 6 suggests a signaling cascade in which the absence of DKK2 leads to enhanced HNF4A expression, which in turn results in reduced Sox9 expression and hence reduced expression of Paneth cell properties. It is therefore crucial that the authors perform in vivo (splenic organoid injection) loss-of-function experiments, knockdown of Sox9 expression in AKP organoids, and Sox9 overexpression experiments in AKP/Dkk2 KO organoids to demonstrate Sox9 as the central downstream transcription factor regulating liver CRC metastasis.

(d) Given the previous description of the role of DKK2 in primary CRC, it is important to define the step of liver metastasis affected by Dkk2 deficiency in the metastasis model. Does it affect extravasation, liver survival, etc.?

Human data:

Can the authors address whether the expression of Dkk2 changes in human CRC and whether mutations in Dkk2 are correlated with metastatic disease or CRC stage?

Bioinformatic analysis

GEO repositories remain not open (at the time of the re-review) and SRA links for raw data are still unavailable. Without access to raw data, it is not possible to verify the analyses or fully assess the results. A part of the article was made by re-analyzing public data so the authors should make even the raw available and not just the count tables

<https://doi.org/10.7554/eLife.97279.2.sa2>

Reviewer #2 (Public review):

Summary:

The authors propose that DKK2 is necessary for the metastasis of colon cancer organoids. They then claim that DKK2 mediates this effect by permitting the generation of lysozyme-positive Paneth-like cells within the tumor microenvironmental niche. They argue that these lysozyme-positive cells have Paneth-like properties in both mouse and human contexts. They then implicate HNF4A as the causal factor responsive to DKK2 to generate lysozyme-positive cells through Sox9.

Strengths:

The use of a genetically defined organoid line is state-of-the-art. The data in Figure 1 and the dependence of DKK2 for splenic injection and liver engraftment, as well as the long-term effect on animal survival, are interesting and convincing. The rescue using DKK2 administration for some of their phenotype in vitro is good. The inclusion and analysis of human data sets help explore the role of DKK2 in human cancer and help ground the overall work in a clinical context.

Remaining Weaknesses after revision:

(1) The authors have effectively explained the regulation of HNF4A at both mRNA and protein levels. To further strengthen their findings, I recommend using CRISPR technology to generate DKK2 and HNF4A double knockout organoids. This approach would allow the authors to investigate whether the AKP liver metastasis is restored in the double knockout condition. Such an experiment would provide more direct evidence that HNF4A protein stabilization is the crucial mechanism for liver metastasis suppression following DKK2 knockout.

<https://doi.org/10.7554/eLife.97279.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:**Reviewer #1 (Public Review):***Summary:*

The authors addressed the influence of DKK2 on colorectal cancer (CRC) metastasis to the liver using an orthotopic model transferring AKP-mutant organoids into the spleens of wild-type animals. They found that DKK2 expression in tumor cells led to enhanced liver metastasis and poor survival in mice. Mechanistically, they associate Dkk2-deficiency in donor AKP tumor organoids with reduced Paneth-like cell properties, particularly Lz1 and Lz2, and defects in glycolysis. Quantitative gene expression analysis showed no significant changes in Hnf4a1 expression upon Dkk2 deletion. Ingenuity Pathway Analysis of RNA-Seq data and ATAC-seq data point to a Hnf4a1 motif as a potential target. They also show that HNF4a binds to the promoter region of Sox9, which leads to LYZ expression and upregulation of Paneth-like properties. By analyzing available scRNA data from human CRC data, the authors found higher expression of LYZ in metastatic and primary tumor samples compared to normal colonic tissue; reinforcing their proposed link, HNF4a was highly expressed in LYZ+ cancer cells compared to LYZ- cancer cells.

Strengths:

Overall, this study contributes a novel mechanistic pathway that may be related to metastatic progression in CRC.

Weaknesses:

The main concerns are related to incremental gains, missing in vivo support for several of their conclusions in murine models, and missing human data analyses. Additionally, methods and statistical analyses require further clarification.

Main comments:

(1) Novelty

The authors previously described the role of DKK2 in primary CRC, correlating increased DKK2 levels to higher Src phosphorylation and HNF4a1 degradation, which in turn enhances LGR5 expression and "stemness" of cancer cells, resulting in tumor progression (PMID: 33997693). A role for DKK2 in metastasis has also been previously described (sarcoma, PMID: 23204234).

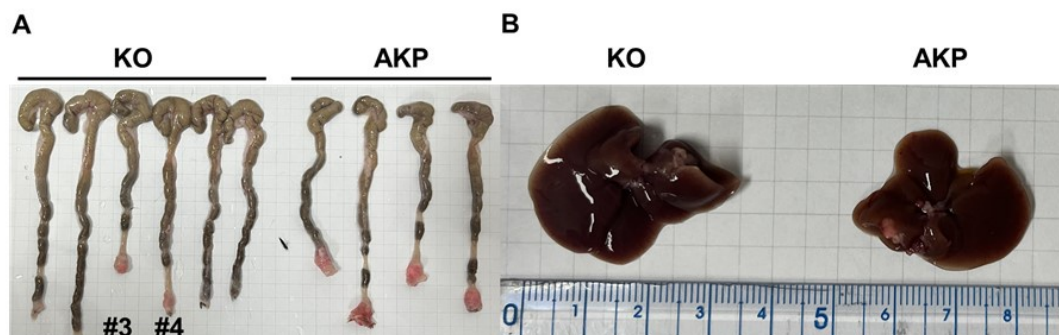
(2) Mouse data

a) The authors analyzed liver mets, but the main differences between AKT and AKP/Dkk2 KO organoids could arise during the initial tumor cell egress from the intestinal tissue (which cannot be addressed in their splenic injection model), or during pre-liver stages, such as endothelial attachment. While the analysis of liver mets is interesting, given that Paneths cells play a role in the intestinal stem cell niche, it is questionable whether a study that does not involve the intestine can appropriately address this pathway in CRC metastasis.

We value the reviewer's comment that the splenic injection model cannot represent metastasis from the primary tumors, intravasation and extravasation. Therefore, we performed the orthotopic transplantation of AKP and KO organoids into the colon directly then, tested metastasis of cancer.

Author response image 1.

Primary tumor formation and liver metastasis by orthotopic transplantation of AKP or KO colon cancer organoids. 6-8 week-old male C57BL/6J mice were treated with 2.5% DSS dissolved in drinking water for 5 days, followed by regular water for 2 days to remove gut epithelium. After recovery with the regular water, the colon was flushed with 1000 μ l of 0.1% BSA in PBS. Then, 200,000 dissociated organoid cells in 200 μ l of 5% Matrigel and 0.1% BSA in PBS were instilled into the colonic luminal space. After infusion, the anal verge was sealed with Vaseline. 8 weeks after transplantation, the mice were sacrificed to measure primary tumor formation and liver metastasis.



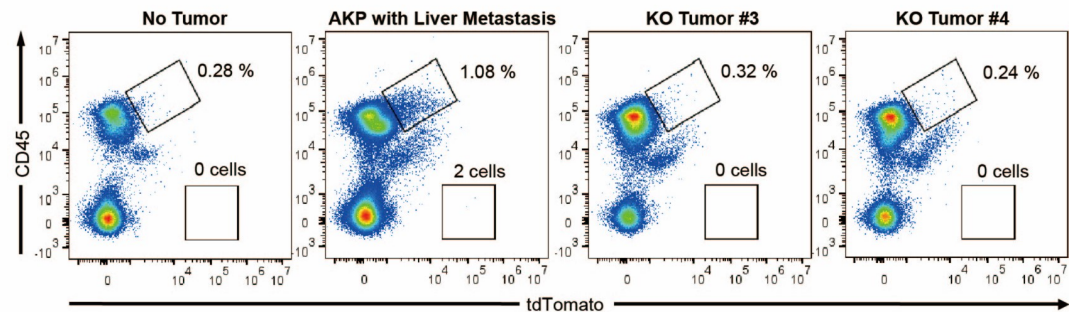
As a result, 4 out of 6 mice in the control group successfully formed colorectal primary tumors whereas only 2 out of 6 mice showed primary tumor formation in the KO group (Author response image 1A). The size of tumors was reduced by about half (10-12 mm to 5-7 mm). Only one AKP mouse developed metastasized nodules in the liver (Author response image 1B). Next, to measure the circulating tumor cells, we harvested at least 500 μ l of bloods from the portal vein and then analyzed tdTomato-positive tumor cells (Author response image 2).

Flow cytometry analysis of PBMCs showed the presence of tdTomato^{hi}CD45⁻ cells as well as tdTomato^{mid}CD45⁺ cells in 2 out of 6 AKP mice, while no tdTomato-positive cells were observed in the PBMCs of KO organoid-transplanted mice.

Due to the limited numbers of mice showed primary and metastatic tumor formation, we cannot provide a statistic analysis of DKK2-mediated metastasis. However, our revised data indicate a trend that DKK2 KO reduced primary tumor formation, the number of circulating tumor cells and liver metastasis. This trend is consistent with our previous report in the *iScience* paper, which showed that DKK2 KO reduced AOM/DSS-induced polyp formation about 60 % and decreased metastasis in the splenic injection model system in this manuscript. Further studies are necessary to confirm this trend and to provide the underlying mechanisms of intravasation and extravasation of circulating tumor cells.

Author response image 2.

Flow cytometry analysis of tdTomato⁺ circulating colon tumor cells in PBMCs. PBMCs were harvested via the portal vein after euthanasia. CD45 and tdTomato were analyzed by flow cytometry.



b) The overall number of Paneth cells found in the scRNA-seq analysis of liver mets was strikingly low (17 cells, Figure 3), and assuming that these cells are driving the differences seems somewhat far-fetched. Adding to this concern is inappropriate gating in the flow plot shown in Figure 6. This should be addressed experimentally and in the interpretation of data.

We appreciate for reviewer's comments to clarify this point. Since the number of LYZ⁺ cells is low in our scRNA-seq analysis, we performed flow cytometry in Figure 6H showing the clear population expressing LYZ in the same splenic injection model of metastasis. Figure 6H is a representative image of triplicates for each group and we performed this experiment three times, independently. As suggested, we changed the graph format and updated the gating and statistical analysis in Fig 6H and 6I. This *in vivo* result confirmed our *in vitro* data showing that DKK2 KO reduced LYZ⁺ cells while increase the HNF4a1 proteins.

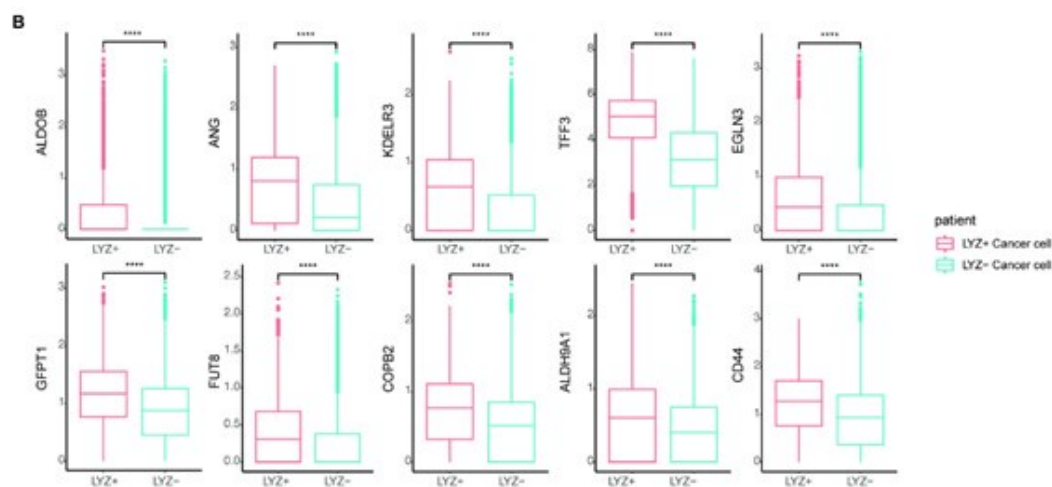
c) Figures 3, 5, and 6 show the individual gene analyses with unclear statistical data. It seems that the p-values were not adjusted, and it is unclear how they reached significance in several graphs. Additionally, it was not stated how many animals per group and cells per animal/group were included in the analyses.

In Fig. 3, mouse scRNA-seq data were generated from pooled cancer samples from 5 animals per group. The Wilcoxon signed-rank test was performed for each gene and/or regulon activity. Since multiple testing adjustments were not performed, a p-value adjustment is neither needed nor applicable..

In Fig. 5, human data were analyzed. Cells from the same sample are dependent, but differential gene expression (DEG) analysis typically calculates statistics under the assumption that they are independent. This assumption may explain the low p-values observed in our data. To address this issue, we applied pseudobulk DEG analysis to our human single-cell data. Even after correcting for statistical error, we confirmed that the genes of interest still exhibited significantly different expression patterns (Author response image 3).

Author response image 3.

Pseudobulk DEG analysis confirmed the differential expression genes of interest.



In Fig.6H-6I, the number of animals per group is provided in the figure legend.

d) Figure 6 suggests a signaling cascade in which the absence of DKK2 leads to enhanced HNF4A expression, which in turn results in reduced Sox9 expression and hence reduced expression of Paneth cell properties. It is therefore crucial that the authors perform in vivo (splenic organoid injection) loss-of-function experiments, knockdown of Sox9 expression in AKP organoids, and Sox9 overexpression experiments in AKP/Dkk2 KO organoids to demonstrate Sox9 as the central downstream transcription factor regulating liver CRC metastasis.

Sox9 is a well-established marker gene for Paneth cell formation in the gut. Therefore, overexpression or knockout of the Sox9 gene would result in either an increase or decrease in Paneth cells in the organoids. We believe that the suggested experiments fall outside the scope of this manuscript. Instead, we demonstrated the change in the Paneth cell differentiation marker, Sox9, in the presence or absence of DKK2.

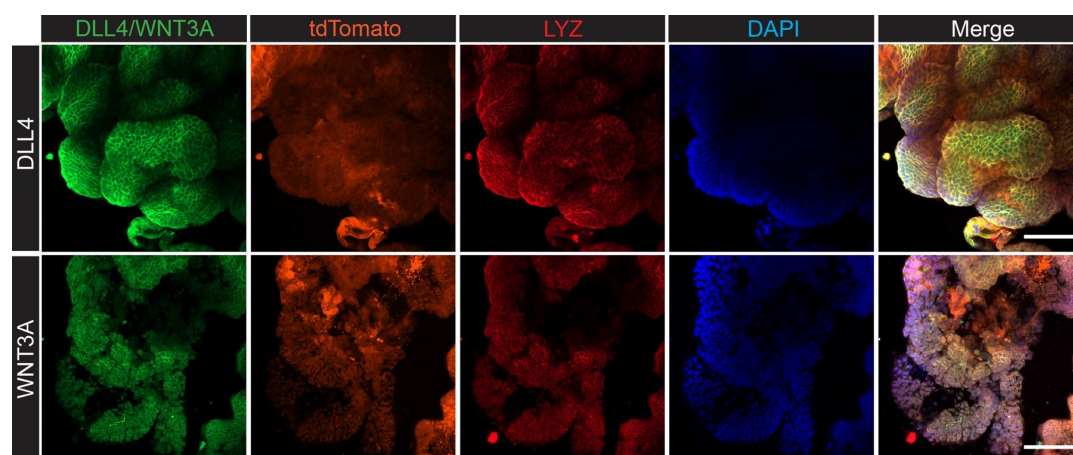
e) Given the previous description of the role of DKK2 in primary CRC, it is important to define the step of liver metastasis affected by Dkk2 deficiency in the metastasis model. Does it affect extravasation, liver survival, etc.?

We appreciate the reviewer's insights and perspectives. Regarding liver survival, it is well known that stem cell niche formation is a critical step for the outgrowth of metastasized cancer cells (Fumagalli et al. 2019, Cell Stem Cell). LYZ+ Paneth cells are recognized as stem cell niche cells in the intestine, and human scRNA-seq data have shown that LYZ+ cancer cells express stem cell niche factors such as Wnt and Notch ligands. To determine whether LYZ+

cancer cells act as stem cell niche cells, we performed confocal microscopy to assess whether LYZ+ cancer cells express WNT3A and DLL4 in AKP organoids (Author response image 4). The results show that LYZ labeling co-localizes with DLL4 and WNT3A expression, while the organoid reporter tdTomato is evenly distributed. Additionally, our in vitro and in vivo data indicate that DKK2 deficiency leads to a reduction of LYZ+ cancer cells, which may contribute to stem cell niche formation. Based on these findings, we propose that DKK2 is an essential factor for stem cell niche formation, which is required for cancer cell survival in the liver during the early stages of metastasis. Although our revised data confirmed the trend that DKK2 deficiency decreases liver metastasis, we have not yet determined whether DKK2 is involved in extravasation. This research topic should be addressed in future studies.

Author response image 4.

Confocal microscopy analysis for lysozyme (LYZ) and Paneth cell-derived stem cell niche factors, WNT3A and DLL4 in AKP colon cancer organoids.



The method is described in the supplemental information. The list of antibodies used: DLL4 (delta-like 4) Polyclonal Antibody (Invitrogen, PA5-85931), WNT3A Polyclonal Antibody (Invitrogen, PA5-102317), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Invitrogen, A-11008), Anti-Lysozyme C antibody (H-10, Santacruz, sc-518083), Goat anti-Mouse IgM (Heavy chain) Secondary Antibody, Alexa Fluor™ 647 (Invitrogen, A-21238).

(3) Human data

Can the authors address whether the expression of Dkk2 changes in human CRC and whether mutations in Dkk2 are correlated with metastatic disease or CRC stage?

The human data were useful in identifying the presence of LYZ+ cancer cells with Paneth cell properties. However, due to the limited number of late-stage patient samples with high DKK2 expression, the results were not statistically significant. Nevertheless, the trend suggests a positive correlation between DKK2 expression and the malignant stage of CRC.

(4) Bioinformatic analysis

The authors did not provide sufficient information on bioinformatic analyses. The authors did not include information about the software, cutoffs, or scripts used to make their analyses or output those figures in the manuscript, which challenges the interpretation and assessment of the results. Terms like "Quantitative gene expression analyses" (line 136) "visualized in a Uniform Approximation and Projection" (line 178) do

not explain what was inputted and the analyses that were executed. There are multiple forms to align, preprocess, and visualize bulk, single cell, ATAC, and ChIP-seq data, and depending on which was used, the results vary greatly. For example, in the single-cell data, the authors did not inform how many cells were sequenced, nor how many cells had after alignment and quality filtering (RNA count, mt count, etc.), so the result on Paneth+ to Goblet+ percent in lines 184 and 185 cannot be reached because it depends on this information. The absence of a clustering cutoff for the single-cell data is concerning since this greatly affects the resulting cluster number (<https://www.nature.com/articles/s41592-023-01933-9>). The authors should provide a comprehensive explanation of all the data analyses and the steps used to obtain those results.

We apologize for the insufficient information. Below, we provide detailed information on the data analyses, which are also available in the GEO database (Bulk RNA-seq: GSE157531, ATAC-seq: GSE157529, ChIP-seq: GSE277510). Methods are updated in the current version of supplemental information.

(5) Clarity of methods and experimental approaches

The methods were incomplete and they require clarification.

We've updated our methods as requested by the reviewer.

Reviewer #2 (Public Review):

Summary:

The authors propose that DKK2 is necessary for the metastasis of colon cancer organoids. They then claim that DKK2 mediates this effect by permitting the generation of lysozyme-positive Paneth-like cells within the tumor microenvironmental niche. They argue that these lysozyme-positive cells have Paneth-like properties in both mouse and human contexts. They then implicate HNF4A as the causal factor responsive to DKK2 to generate lysozyme-positive cells through Sox9.

Strengths:

The use of a genetically defined organoid line is state-of-the-art. The data in Figure 1 and the dependence of DKK2 for splenic injection and liver engraftment, as well as the long-term effect on animal survival, are interesting and convincing. The rescue using DKK2 administration for some of their phenotype in vitro is good. The inclusion and analysis of human data sets help explore the role of DKK2 in human cancer and help ground the overall work in a clinical context.

Weaknesses:

In this work by Shin et al., the authors expand upon prior work regarding the role of Dickkopf-2 in colorectal cancer (CRC) progression and the necessity of a Paneth-like population in driving CRC metastasis. The general topic of metastatic requirements for colon cancer is of general interest. However, much of the work focuses on characterizing cell populations in a mouse model of hepatic outgrowth via splenic transplantation. In particular, the concept of Paneth-like cells is primarily based on transcriptional programs seen in single-cell RNA sequencing data and needs more validation. Although including human samples is important for potential generality, the strength could be improved by doing immunohistochemistry in primary and metastatic lesions for Lyz+ cancer cells. Experiments that further bolster the causal role of Paneth-like CRC cells in metastasis are needed.

Recommendations for the Authors:

Reviewing Editor (Recommendations for the Authors):

Here we note several key concerns with regard to the main conclusions of the paper. Additional experiments to directly address these concerns would be required to substantially update the reviewer evaluation.

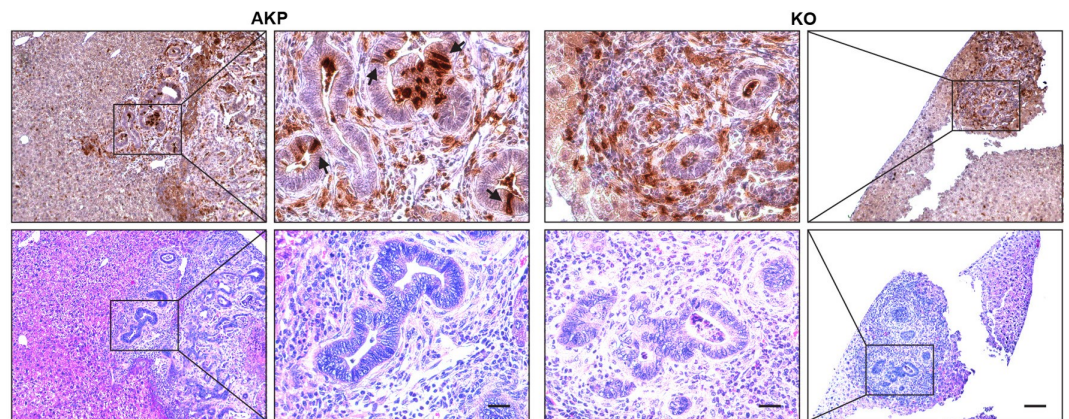
(1) Demonstration of a causal role of Paneth-like cells in CRC metastasis, for example by sorting the Paneth-like cells - either by the markers they identified in the subsequent single cell or by scatter - to establish whether the frequency of the Paneth-like cells in a culture of organoids is directly correlated with tumorigenicity and engraftment.

We sincerely appreciate the reviewing editor's comment. First, as previously reported (Shin et al., iScience 2021), there is no difference in proliferation between WT and KO during in vitro organoid culture or in vivo colitis-induced tumors. However, DKK2 deficiency led to morphological changes, which we analyzed using bulk RNA-seq. As described in the manuscript, Paneth cell marker genes, such as Lysozymes and defensins, were significantly reduced in DKK2 KO AKP organoids.

Due to the nature of these markers, it is technically challenging to isolate live LYZ+ cancer cells. To address this issue in the future, we plan to develop organoids that express a reporter gene specific for Paneth cells. In this manuscript, we demonstrated a correlation between DKK2 and the formation of LYZ+ cancer cells. In both the splenic injection model (Fig. 1) and the orthotopic transplantation model (Fig. R1-R2), we observed that transplantation of cancer organoids with reduced numbers of LYZ+ cells (KO organoids) led to decreased metastatic tumor formation. The number of LYZ+ cells in KO-transplanted mice remained low in liver metastasized tumor nodules (Fig. 6H-I6). Immunohistochemistry further confirmed that LYZ+ cancer cells were barely detectable in KO samples (Author response image 5). These data suggest that DKK2 is essential for the formation of LYZ+ cancer cells, which are necessary for outgrowth following metastasis.

Author response image 5.

Histology of Lysozyme positive cells in metastasized tumor nodules in liver of colon cancer organoid transplanted mice. Immunohistochemistry of Lysozyme positive Paneth-like cells in liver metastasized colon cancer (Upper panels, DAB staining). Identification of tumor nodules by H&E staining (lower panels, Scale bar = 100 µm). Magnified tumor nodules are shown in the 2nd and 3rd columns (Scale bar = 25 µm). Arrows indicate Lysozyme positive Paneth like cells in tumor epithelial cells. Infiltration of Lysozyme positive myeloid cells is detected in both AKP and KO tumor nodules. AKP: Control colon cancer organoids carrying mutations in Apc, Kras and Tp53 genes. KO: Dkk2 knockout colon cancer organoids



(2) Further characterization of Lyz+/Paneth-like cells to further the authors' argument for the unique function that they have in their tumor model. Specifically, do the cells with Paneth-like cells secrete Wnt3, EGF, Notch ligand, and DII4 as normal Paneth cells do?

We appreciate the reviewing editor's comment. In response, we performed confocal microscopy analysis to examine the protein levels of LYZ, Wnt3A, and DLL4 in AKP colon cancer organoids (Author response image 4). The data presented above show that LYZ+ cancer cells express both Wnt3A and DLL4, suggesting that LYZ+ colon cancer cells may function similarly to Paneth cells, which are stem cell niche cells. Furthermore, using the Panglao database, we demonstrated that LYZ+/Paneth-like cells exhibit typical Paneth cell properties in human scRNA-seq data (Fig. 4 and Fig. 5). These findings suggest that LYZ+ colon cancer cells possess Paneth cell properties.

(3) Experiments to test metastasis, ideally from orthotopic colonic tumors, to ensure phenotypes aren't restricted to the splenic model of hepatic colonization and outgrowth used at present.

We are in agreement with the reviewing editor and reviewers, which is why we conducted the orthotopic transplantation experiment. However, we encountered challenges in establishing this model effectively. After multiple trials, we observed that many mice did not form primary tumors, and the variability, particularly in metastasis, was difficult to control. Only a few AKP-transplanted mice developed liver metastasis. The representative revision data have been provided above. Nevertheless, we believe that this model needs further improvement and optimization to reliably study metastasis originating from primary tumors.

(4) To generalize claims to human cancer, the authors should test whether loss of DKK2 impacts LYZ+ cancer cells in human organoids and affects their engraftment in immunodeficient mice compared to control. Another more correlative way to validate the LYZ+ expression in human colon cancer would be to stain for LYZ in metastatic vs. primary colon cancer, expecting metastatic lesions to be enriched for LYZ+ cells.

We agree with your point, and this will be addressed in future studies.

(5) Clarifying inconsistencies regarding effect of DKK2 loss on HNF4A (Figure 1E vs Figure 6I).

In Figure 1 E, we measured the mRNA levels of HNF4A in metastasized foci by qPCR while in Figure 6I, we measured the protein level of HNF4A by flow cytometry. Recent studies, including our previous report, have shown that HNF4A protein levels are regulated by

proteasomal degradation mediated by pSrc (Mori-Akiyama *et al.* 2007, *Gastroenterology*, Bastide *et al.* 2007, *Journal of Cell Biology*, Shin *et al.* 2021 *iScience*). Consequently, while the mRNA levels remained unchanged in Fig. 1E, we observed a reduction of HNF4A protein levels in Figure 6I.

(6) Addressing concerns about statistics and reporting as outlined by Reviewer 1.

Thank you very much for your assistance in improving our manuscript. The updates have been incorporated as detailed above.

These are the central reviewer concerns that would require additional experimentation to update the editorial summary. Other concerns should be addressed in a revision response but do not require additional experimentation.

Reviewer #1 (Recommendations For The Authors):

Specific comments:

- *Do Dkk2-KO organoids grow normally?*

Yes, *in vitro*.

Since the authors reported on the effects of Dkk2 in the induction/maintenance of the Paneth cell niche, changes in AKP organoid numbers of growth rate between Dkk2-WT and KO would be an expected outcome.

Disruption of Paneth cell formation in normal organoids is expected to alter growth. However, DKK2 KO in colon cancer organoids with mutations in the *Apc*, *Kras*, and *Tp53* genes exhibits growth rates and organoid sizes similar to those of WT AKP controls. In contrast to *in vitro* observations, we observed a significant reduction in metastasized tumor growth *in vivo*. Further analyses of factors derived from LYZ+ cancer cells will help address the discrepancy in DKK2's absence between *in vitro* and *in vivo* conditions.

- *Figure 1:*
 - *Panel C: The legend indicates what c.p. stands for.*

c.p.m. stands for count per minutes for *in vivo* imaging analysis. This has been updated in the Figure legend.

- *Panel E: Please comment on the possible underlying reasons for the lack of change in HNF4a1 levels.*

This has been updated in response to the reviewing editor's comment (5) above.

- *Panel E: Number of mice from which isolated cancer nodules were harvested.*

Total mice per group were 5. This has been updated in the legend.

- *Figure 2:*
 - *Suggestion: Panel A should be presented in Figure 1 since Dkk2 KO organoids are already used in Figure 1.*

We added this to present the recovery of DKK2 by adding recombinant DKK2 proteins in Fig.2.

- Panel B: Please explain why these genes are marked in blue.

It has been described in the legend. "Paneth cell marker genes are highlighted as blue circles (AKP=3 and KO=5 biological replicates were analyzed)."

• Figure 3:

- Indicate the number of cells recovered from AKP vs. KO mice (since liver metastasis was already reduced in KO mice). This should be shown in a UMAP.

- Panel A: 4th line in the pathways, correct "Singel" typo.

We appreciate your correction. It has been fixed.

- Panel A: There are multiple versions of PanglaoDB with different markers; a list of all that was used to determine cell type should be provided.

- Panel C: Bar value for the WNT pathway is not displayed, and there is no legend to indicate the direction of the analysis (that is, AKPvsKO or KOvsAKP).

It is KOvsAKP, described in the figure legend.

- Panel C: Ingenuity pathway analysis is not a good tool to look at this type of result because it does not include the gene fold changes in the analysis, so it only provides a Z-score of the presence of that pathway and not the degree it is increased or fold changes - recommend substituting any type of GSEA analysis, such as fgsea. -o Panel D: the term "Patient" to refer to mice is confusing. Use "Mice" or "Treatment" or "Condition" instead.

Corrected

- Panel D: Information about the number of mice per group, cells per animal (or liver let) used, and additional clarification about the statistical analysis used is required, as differences shown in this panel appear subtle given the standard variation in each group. Box plots need to show individual/raw values.

• Figure 4:

- Panel E: It would be helpful to show the cutoff lines for the Paneth cell score and Lyz expression in the graphs.

It has been updated in response to the reviewer's request.

• Figure 5:

- Panel B: again, information about the number of "patients" or cells used and clarification about the statistical analysis used is required as the display of data generates concerns about the distribution within groups. Box plots need to show individual/raw values

It has been updated in response to the reviewer's request.

• Figure 6:

- Panel A: Add a legend to inform the direction of the process (e.g., red, activation, blue, repression). We noticed the Yap1 bar data had no color. Is there a reason for that?

Please explain this point in the revised manuscript.

Red color added for the Yap1.

- Panel A: Ingenuity pathway analysis is not a good tool to look at this type of results because it does not include the gene Foldchanges in the analysis, so it only provides a Z-score of the presence of that pathway and not the degree it is increased or not. I recommend substituting any type of GSEA analysis, such as fgsea.
- Panels A&B: Again, only p-value scores were provided, while fold changes are necessary to define the ratio of presence increase of normal vs. AKP.
- Panel D: No raw or pre-processed ChIP-seq data was provided. Additionally, please indicate exactly the genome location (it seems the image was edited from a raw made on UCSC genome browser-it should be remade by adding coordinates and other important information (genes around, epigenetic, etc.).
- Panel H/I: Flow cytometry gating is inappropriate, as its catching cells are negative for LYZ in both AKP and KO cells, resulting in an overestimation of the number of Lyz cells. Gating should specifically select very few LYZ-positive cells in the top/left quadrant.

The updates have been made, and the statistical data have been re-analyzed.

- Panel J: Information about the number of animals/organoids or cells used and clarification about the statistical analysis used is required, as the display of data generates concerns about the distribution within groups. Box plots need to show individual/raw values.
- Overall:
- A supplementary table with all the sequenced libraries and their depth, read length/cell count should be provided.

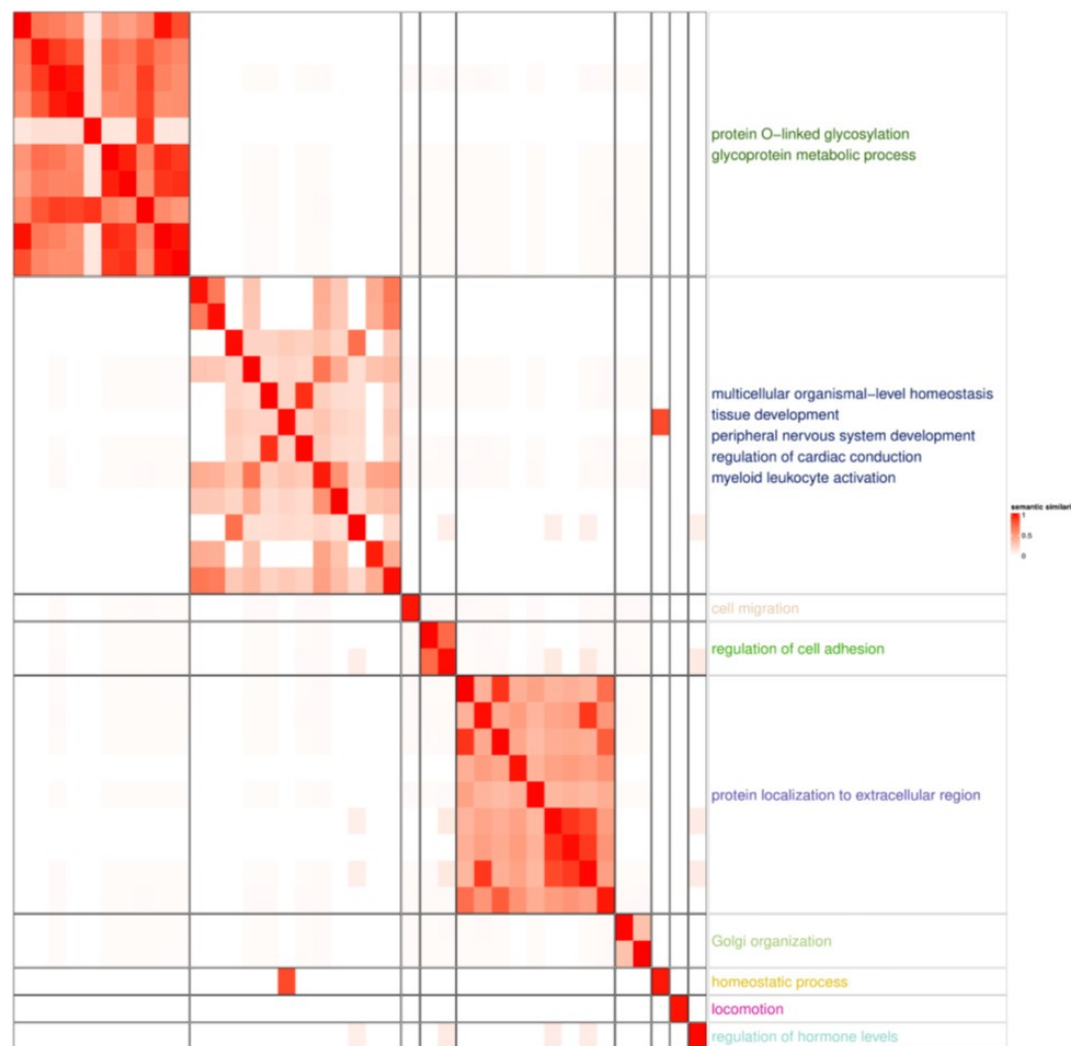
All of the information is now available in the GEO database. We used previously published human epithelial datasets for human single cell analysis (Joanito*, Wirapati*, Zhao*, Nawaz* et al, Nat Genetics, 2022, PMID: 35773407).

- The Hallmark Geneset used is very broad, and the authors should confirm the results on GO bp.

Using Gene Ontology biological processes (GO bp), we observed that glycolysis-related genes were enriched in our newly described cell population, although the adjusted p-value did not exceed 0.05.

Author response image 6

GSEA with GOBP pathway highlighted glycoprotein and protein localization to extracellular region, both of which are related Paneth cell functions. Paneth cells secrete α -defensins, angiogenin-4, lysozyme and secretory phospholipase A2. The enriched glycoprotein process and protein localization not extracellular region reflect the characteristics of Paneth cells.



- qPCR is not a good way to confirm sequencing results; while PCR data is pre-normalized, sequencing is normalized only after quantification, so results on 6 E and F should be shown on the sequencing data.

The expression level of Sox9 is relatively low. In our bulk RNA-seq data, the averages for Sox9 in AKP versus DKK2 KO are 28.2 and 25.1, respectively. While there is a similar trend, the difference is not statistically significant in this dataset, and we did not include an experimental group for reconstitution. Therefore, we conducted qPCR experiments for the reconstitution study by adding recombinant DKK2 (rmDKK2) protein to the culture. Furthermore, it is well established that Sox9 is an essential transcription factor for the formation of LYZ⁺ Paneth cells. Based on this, we assessed the levels of LYZ and Sox9 using qPCR and confocal microscopy in the presence or absence of DKK2.

• Edits in the text:

- There are several typographical errors. Specific suggestions are provided below.
- Line 43: "Chromatin immunoprecipitation followed by sequencing analysis," state analysis of what cells before continuing with "revealed..." revealed...
- Line 77: Recent findings have identified

- Line 138: *were reduced in KO tumor samples* → rephrase to clarify "KO-derived liver tumors"
- Line 167: *Recombinant mouse DKK2 protein treatment in KO organoids partially rescued this effect. Add "partially" since adding rmDkk2 didn't fully restore Lyz1 and Lyz2 levels.*
- Line 185-187: *the authors should not reference Figure 6 because it has not been introduced yet.*
- Line 198-199: *The authors claimed a correlation between Dkk2 expression and Lgr5 expression; however, the graph presented in Figure 3B does not indicate this. The R-value was 0.11, which does not indicate a correlative expression between these genes.*
- Line 232-233: *the authors need to show any connection to Dkk2 gene expression in human samples in order to draw that conclusion.*
- Line 294: *expression, leading to the formation*
- Line 347: *Wnt ligand (correct Wng typo)*

We have modified our manuscript in accordance with the reviewer's suggestions.

Reviewer #2 (Recommendations For The Authors):

Specific criticisms/suggestions:

Author claim 1: Dkk2 is necessary for liver metastasis of colon cancer organoids. This model is one of hepatic colonization and eventual outgrowth and not metastasis. Metastasis is optimally assessed using autochthonous models of cancer generation, with the concomitant intravasation, extravasation, and growth of cancer cells at the distant site. The authors should inject their various organoids in an orthotopic colonic transplantation assay, which permits the growth of tumors in the colon, and they can then identify metastasis in the liver that results from that primary cancer lesion (i.e., to better model physiologic metastasis from the colon to liver).

The data of orthotopic colonic transplantation data has been provided above (Author response images 1 and 2).

Author claim 2: DKK2 is required for the formation of lysozyme-positive cells in colon cancer.

It would greatly strengthen the authors' claim if supraphysiologic or very high amounts of DKK2 enhance CRC organoid line engraftment (i.e., the specific experiment being pre-treatment with high levels of DKK2 and immediate transplantation to see a number of outgrowing clones). If DKK2 is causal for the engraftment of the tumors, increased DKK2 should enhance their capacity for engraftment.

Paneth cells have physical properties permitting sorting and are readily identifiable on flow cytometry. The authors should demonstrate increased tumorigenicity and engraftment by sorting the Paneth-like cells-either by the markers they identified in the subsequent single cell or by scatter to establish whether the frequency of the Paneth-like cells in a culture of organoids is directly correlated with engraftment potential.

Further characterization of the Paneth-like cells would help further the authors' argument for the unique function that they have in their tumor model. Specifically, do the cells with Paneth-like cells secrete Wnt3, EGF, Notch ligand, and DII4 as normal

Paneth cells do? Immunofluorescence, sorting, or western blots would all be reasonable methods to assess protein levels in the sorted population.

This has been performed and provided above (Author response images 1 and 3)

Author claim 3: Lysozyme (LYZ)+ cancer cells exhibit Paneth cell properties in both mouse and human systems.

For the claim to be general to human cancer, the author should demonstrate that loss of DKK2 impacts LYZ+ cancer cells in human organoids and affects their engraftment in immunodeficient mice compared to control. Another more correlative way to validate the LYZ+ expression in human colon cancer would be to stain for LYZ in metastatic vs. primary colon cancer, expecting metastatic lesions to be enriched for LYZ+ cells.

The claims on the metabolic function of Paneth-like cells need more clarification. Do the cancer cells with Paneth features have a distinct metabolic profile compared to the other cell populations? The authors should address this through metabolic characterization of isolated LYZ+ cells with Seahorse or comparison of Dkk2 KO to WT organoids (i.e., +/-LYZ+ cancer cell population).

To address this question, we need to develop organoids with a Paneth cell reporter gene. We appreciate the reviewer's comment, and this should be pursued in future studies.

Author claim 4: HNF4A mediates the formation of Lysozyme (Lyz)-positive colon cancer cells by DKK2.

The authors implicate HNF4A and Sox9 as causal effectors of the Paneth-like cell phenotype and subsequent metastatic potential. There appears to be some discordance regarding the effect of DKK2 loss on HNF4A. In Figure 1E, the authors show that gene expression in metastatic colon cancer cells for HNF4A in DKK2 knockout vs AKP control is insignificant. However, in Figure 6I, there is a highly significant difference in the number of HNF4A positive cells, more than a 3-fold percentage difference, with a p-value of <0.0001. If there is the emergence of a rare but highly expressing HNF4A cell type that on aggregate bulk expression leads to no difference, but sorts differentially, why is it not identified in the single-cell data set? These data together are highly inconsistent with regards to the effect of DKK2 on HNF4A and require clarification.

Previous studies have demonstrated that HNF4A is regulated by proteasomal degradation mediated by pSrc. As a result, the mRNA level of HNF4A remains unchanged, while the protein level is significantly reduced in colon cancer cells. DKK2 KO leads to decreased Src phosphorylation, resulting in the recovery of HNF4A protein levels. This explains why HNF4A cannot be detected in scRNA-seq datasets, which measure mRNA. We have shown this in our previous report. In this manuscript, based on ChIP-seq data using an anti-HNF4A monoclonal antibody, as well as confocal microscopy and qPCR data for the Sox9 gene, we propose that HNF4A acts as a regulator of cancer cells exhibiting Paneth cell properties.

<https://doi.org/10.7554/eLife.97279.2.sa0>