Dickkopf-2 regulates the stem cell marker LGR5 in colorectal cancer via HNF4α1
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SUMMARY
Enhanced stemness in colorectal cancer has been reported and it contributes to aggressive progression, but the underlying mechanisms remain unclear. Here we report a Wnt ligand, Dickkopf-2 (DKK2) is essential for developing colorectal cancer stemness. Genetic depletion of DKK2 in intestinal epithelial or stem cells reduced tumorigenesis and expression of the stem cell marker genes including LGR5 in a model of colitis-associated cancer. Sequential mutations in APC, KRAS, TP53, and SMAD4 genes in colonic organoids revealed a significant increase of DKK2 expression by APC knockout and further increased by additional KRAS and TP53 mutations. Moreover, DKK2 activates proto-oncogene tyrosine-protein kinase Src followed by increased LGR5 expressing cells in colorectal cancer through degradation of HNF4α1 protein. These findings suggest that DKK2 is required for colonic epithelial cells to enhance LGR5 expression during the progression of colorectal cancer.

INTRODUCTION
Colorectal cancer is the third most common cancer in the world and the second leading cause of cancer related death (Bray et al., 2018). About 80% of colorectal cancer patients have mutations in the adenomatous polyposis coli (APC) gene, a negative regulator of canonical Wnt signaling (Phelps et al., 2009). APC mutations in stem cells initiate intestinal tumorigenesis (Barker et al., 2009). Barker et al. has identified LGR5 as an intestinal stem cell marker gene (Barker et al., 2007). LGR5 positive intestinal stem cell-specific APC conditional knockout mice showed spontaneous tumor formation whereas APC deletion in non-stem cells was rarely tumorigenic (Barker et al., 2009). Lineage tracing studies further confirmed LGR5 positive stem cells as cancer stem cells in the gut (Schepers et al., 2012). Additional oncogenic mutations in KRAS, TP53 and SMAD4 in epithelial cells promote colorectal carcinogenesis (Vogelstein et al., 1988). Briefly, oncogenic alterations in intestinal stem cells lead to initiation and progression of colorectal cancer.

Recent advances in organoid culture techniques allowed in vitro primary intestinal epithelial cell culture (Sato et al., 2009; Drost and Clevers, 2018). Mutations in APC, KRAS, TP53 and SMAD4 in human intestinal organoids using CRISPR methods provided a better understanding of carcinogenesis in colorectal cancer (Matano et al., 2015). Depletion of LGR5 expressing cancer stem cells in murine primary or metastatic colon cancer cells carrying those mutations have shown the necessity of cancer stem cells in metastatic progression (de Sousa e Melo et al., 2017). However, underlying mechanisms of how LGR5 expressing cancer stem cells arise and are maintained are still elusive.

In this study, we introduce DKK2 as an essential factor for LGR5 expression in colon cancer. We showed increased DKK2 expression in colon tumor epithelial cells. Using colon cancer organoids, we showed a significant increase of DKK2 transcription by triple mutations in APC, KRAS, and TP53 genes. Gain and loss of function studies using normal or cancer organoids revealed a feedforward feedback regulation of DKK2 expression. Mechanistically, we have identified that DKK2 activates proto-oncogene tyrosine-protein kinase Src (c-Src), which then results in HNF4α1 protein degradation followed by enhanced LGR5 expression in colon cancer. Our findings provide a better understanding of the formation of LGR5 expressing cancer stem cells during colorectal cancer progression.
**RESULTS**

**DKK2 is highly expressed in colorectal cancer epithelium and facilitates tumorigenesis**

DKK2 expression is increased about 50-fold in colorectal cancer patients compared to healthy normal controls and polyp-specific DKK2 expression in APC mutant (Apc\(^{min}\)) mice has been reported by in situ hybridization (Matsui et al., 2009; Gregorieff et al., 2005). We have confirmed the elevation of DKK2 in colorectal cancer using colorectal cancer patient samples and a murine colitis-induced tumor model. Transcriptional increase of DKK2 was observed in human colorectal carcinoma RNA sequencing (RNA-seq) data along with increased expression of the stem cell marker gene, LGR5 (Figure 1A). DKK2 expression was increased about 100-fold in tumor tissues compared to untreated controls in the azoxymethane and dextran sodium sulfate (AOM/DSS)-treated colitis-induced tumor model (Figures 1B and 1C). Polyp-specific increase of DKK2 expression was observed in a time course experiment (Figure 1D). Immunohistochemistry analysis for DKK2 showed epithelial expression of DKK2 in murine colitis-induced tumors (Figure 1E). To confirm the pattern of DKK2 expression, we sorted epithelial cells from colitis-induced tumors based on epithelial marker gene (EpCAM) and stem cell marker gene expression (LGR5) using Lgr5\(^{CwERT2-IRES-EGFP}\) reporter mice (Figures S1A and S1B). The sorted LGR5\(^{high}\), LGR5\(^{mid}\), and LGR5\(^{neg}\) populations represent intestinal stem cell populations, transit amplifying cell and Paneth cell populations and differentiated epithelial cell populations, respectively (Sato et al., 2009). Increased expression of DKK2 was shown along with LGR5 expression (Figure 1F). These data demonstrate a significant increase of DKK2 expression and its pattern in colonic epithelium in the colitis-induced tumor model.

Next, in order to investigate the roles of DKK2 in intestinal tumorigenesis, we generated intestinal epithelium-specific DKK2 conditional knockout Villin\(^{lox-}\)-Dkk2\(^{fl/fl}\) mice. Eighty days after AOM/DSS treatment, the number and size of polyps were recorded by colonoscopy. DKK2 knockout in the intestinal epithelium showed about 60% reduction of tumor growth (Figures 1G–1J and S1C). Interestingly, DKK2 knockout does not alter proliferation of colitis-induced tumor cells (Figures S1D and S1E). Rather, the expression of stem cell marker gene LGR5 was reduced in the absence of DKK2 and there was a correlation of expression between DKK2 and LGR5 during colitis-induced tumor development (Figures 1K and 1L). These findings suggest that DKK2 contributes to intestinal tumorigenesis and the elevation of LGR5 expression in colorectal cancer.

**DKK2 depletion modulates stenessness and differentiation of colitis-induced tumor cells**

Using tamoxifen-inducible intestinal stem cell-specific DKK2 knockout (Lgr5\(^{CwERT2-IRES-EGFP, Dkk2^{fl/fl}}\)) mice, we have confirmed that DKK2 expression in LGR5 positive cells and its daughter cells promotes colitis-induced tumorigenesis (Figures 2A–2E). The correlation between DKK2 and LGR5 expression was confirmed as well and those results were not a consequence of the functional loss of one LGR5 promoter allele in the Lgr5\(^{CwERT2-IRES-EGFP, Dkk2^{fl/fl}}\) mice (Figures S1F and S1G).

As an unbiased way to assess the roles of DKK2 in intestinal tumorigenesis, we performed a bulk RNA-seq analysis on colitis-induced polyps from the AOM/DSS-treated Lgr5\(^{CwERT2-IRES-EGFP, Dkk2^{fl/fl}}\) mice. Consistent with the previous Villin\(^{lox-}\)-Dkk2\(^{fl/fl}\) mice data, the expression of LGR5 was decreased in the absence of DKK2 (Figure 2F). Quiescent stem cell marker genes, BMI1 and TERT, were reduced as well whereas the differentiation marker genes, EMP1, KRT20, and SLC26A3 were increased and it has been confirmed with quantitative PCR analysis (Figure 2G). Again, expression of proliferation marker genes such as Ki67 was unaltered in DKK2-deficient polyps (Figure 2G). These results imply that DKK2 regulates stemness and differentiation of epithelial cells in a colitis-induced tumor model.

**DKK2 enhances Lgr5 expression in colonic epithelial cells**

In the canonical Wnt signaling pathway, DKK2 has been introduced as a Wnt antagonist, binding to the common Wnt receptors, LRP5 and LRP6 (Niehrs, 2006). The following studies have reported the agonistic roles of DKK2 in the canonical Wnt pathway in the absence of another Wnt receptor Kremen2 (Mao and Niehrs, 2003). To test the direct effects of DKK2 on colonic epithelial cells, we performed normal colonic organoid culture in a colitis-induced tumor model. Consistent with previous DKK2 treatment further increased DKK2 expression in organoids in a dose- and time-dependent manner indicating the feedforward regulation of its expression (Figures 3C and 3D).
Next, we questioned whether our \textit{in vitro} findings are reproducible in tumor settings. In order to understand the expression conditions of DKK2 during colorectal cancer development, we generated colon cancer organoids carrying various combinations of mutations in \textit{APC}, \textit{KRAS}, \textit{TP53}, and \textit{SMAD4} genes that are commonly observed during colorectal cancer progression (Vogelstein et al., 2013) (Figure 3E). Accumulating mutations in those genes resulted in the elevation of LGR5 expression in colonic organoids (Figure S2A). Expression of DKK2 was detected in \textit{Apc knockout} (A) and \textit{Apc knockout KrasG12D} (AK) mutant organoids (Figure 3F). It was further increased in Apc

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\textbf{Figure 1.} \textbf{DKK2 expression in colon cancers and reduced colitis-induced tumorigenesis in DKK2 deficient mice}

(A) Expression of DKK2 and LGR5 in human colorectal carcinoma and adjacent normal tissues was analyzed using previously deposited RNA-seq data (GSE50760, \(n = 18\) each group, TPM; Transcripts per million).

(B) A schematic diagram of azoxymethane/dextran sodium sulfate (AOM/DSS) treatment schedule.

(C) Quantitative PCR analysis of DKK2 expression in mouse colon tissues or polyps upon AOM/DSS treatment. Each symbol indicates an individual tissue sample.

(D) Expression kinetics of DKK2 in the AOM/DSS-treated wild type C57BL/6 colon tissues measured by quantitative PCR (AD, AOM/DSS, Cy1, 2 and 3; the first, second and third DSS cycle, P, polyps, d80; day 80, Red; polyps, Black; normal tissues). Each dot represents an individual tissue sample.

(E) Representative DKK2 immunohistochemistry in AOM/DSS-induced tumors. Scale bar = 10 \(\mu\)m.

(F) Quantitative analysis of DKK2 expression in the sorted tumor cells based on EGFP signal from AOM/DSS treated \textit{Lgr5-EGFP} reporter mice.

(G) Tumor number and score by colonoscopy in wild type (WT, \(n = 19\)) or \textit{VillinCre-Dkk2fl/fl} knockout (KO, \(n = 17\)) mice on day 80. \(n\) represents the number of independent mice.

(H–J) Representative of colonoscopy, macroscopic view and histology on day 80.

(K) Quantitative gene expression analysis of LGR5 in tumor tissues after AOM/DSS treatment. WT N: wild type normal colon, \(n = 4\), WT T: wild type tumor, \(n = 16\), KO N: Dkk2 knockout normal colon, \(n = 4\), KO T: Dkk2 knockout tumor, \(n = 20\). \(n\) represents the number of tumor tissues.

(L) Correlation of DKK2 and LGR5 relative expression to \textit{Hprt} in tumor samples by Pearson \(r\) test. Each dot represents an individual tissue sample. n.s, not significant, *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\); two-tailed Welch’s test. Error bars indicate mean \(\pm\) s.d. Results are representative of three independent experiments.
knockout KrasG12D and Tp53 knockout triple mutant (AKP) organoids and this expression level was maintained in quadruple mutant (AKPS) organoids with additional knockout in the SMAD4 gene (Figure 3F). Given that information, AKP organoids were used for the following experiments.

Th et i m e c o u r s e x p r e s s i o n o f DKK2 and LGR5 in AKP organoids was analyzed for 10 days after single isolated cells were embedded in 3-dimensional matrigel culture. DKK2 expression increased and reached a plateau at day 8 (Figure S2B). LGR5 expression increased at day 4-6 when spheroids formed then decreased about two-fold and was maintained at day 8-10 when enteroid formation was mostly observed (Figure S2C).

Based on these results, we decided to perform transcriptome analysis at day 8. We then developed DKK2 knockout AKP (KO) organoids using the CRISPR technique. To rule out unwanted results by Cas9-driven random mutations on DKK2, we cloned DKK2 knockout AKP organoids through single cell sorting and sequencing (Figure S2D).

The time course expression of DKK2 and LGR5 in AKP organoids was analyzed for 10 days after single isolated cells were embedded in 3-dimensional matrigel culture. DKK2 expression increased and reached a plateau at day 8 (Figure S2B). LGR5 expression increased at day 4-6 when spheroids formed then decreased about two-fold and was maintained at day 8-10 when enteroid formation was mostly observed (Figure S2C). Based on these results, we decided to perform transcriptome analysis at day 8.
Figure 3. DKK2 enhances Lgr5 expression in intestinal epithelial cells without affecting expression of the canonical Wnt target genes

Normal colonic organoids were cultured in the presence or absence of recombinant DKK2 for 3 days (rmDKK2: Recombinant mouse DKK2 protein).

(A and B) Quantitative gene expression analyses of the canonical Wnt target genes (A) and Lgr5 (B).

(C and D) Dkk2 expression after rmDKK2 treatment in a dose- (C) and time-dependent (D) manner.

(E) A schematic diagram of colon cancer organoid development. Apc, Kras and Tp53 triple (AKP) and plus Smad4 quadruple (AKPS) organoids are shown. Colonic organoids were generated from Lsl-KrasG12D/+Tp53R172H/LSL-TdT mice. AKP triple mutations were acquired by adenoviral transduction of pSECC plasmids carrying Cre, Cas9 and guide RNA targeting Apc to isolated single organoid cells. Mutant tumor organoids were selected during culture in the absence of Wnt3a, EGF and Nutlin recombinant proteins for Apc, Kras and Tp53 mutations, respectively. Additional adenoviral transduction and recombinant TGFβ selection were performed to incorporate Smad4 mutations in order to generate AKPS organoids. LSL: LoxP-Stop-Loxp, TdT: Tandem dimer Tomato.

(F) Dkk2 expression in wild type (WT) colonic organoids and colon cancer organoids carrying Apc mutation (A), Apc and Kras mutations (AK), Apc, Kras and Tp53 mutations (AKP) and Apc, Kras, Tp53 and Smad4 mutations (AKPS).

(G) Expression of the canonical Wnt target genes in Dkk2 knockout AKP (KO) organoids.

(H) Ingenuity pathway analysis of colitis-induced tumor RNA-seq data suggested canonical pathways by Dkk2 knockout. Numbers indicate activation Z score.

(I) Expression of Dkk2, Lgr5, Emp1 and Slc26a3 in KO organoids.

(J) Representative pictures and the percentiles of spheroid versus enteroids in control and KO organoids. Cystic organoids were counted as spheroids while budding shape organoids were counted as enteroids. 20 independent images were analyzed in each group. Scale bar = 50 μm. n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001; two-tailed Welch’s t-test. Error bars indicate mean ± s.d.

(Figure S2E). While the CRISPR technique does not alter target gene transcription, we observed more than 50% reduction in DKK2 expression after knockout, indicating that the positive feedback regulation in DKK2 expression was abolished as well (Figure S2F). In accordance with the above in vitro data using DKK2-treated normal colonic organoids, DKK2 knockout in AKP colon cancer organoids resulted in unchanged expression of the canonical Wnt target genes (Figure 3G). Confocal microscopy and flow cytometry analysis of CTNNB1 in AKP organoids showed that DKK2 knockout did not alter cellular localization and protein levels of CTNNB1 (Figures S2A and S2B). Moreover, ingenuity pathway analysis of the RNA-seq data from colitis-induced polyps showed marginal reduction of the canonical Wnt signaling pathway activity in the absence of DKK2 (Figure 3H). These results imply the possibility of the presence of non-canonical DKK2 signaling.

Although the canonical Wnt target genes were unaffected by DKK2 knockout, reduced Lgr5 expression and enhanced differentiation of intestinal epithelial cells are still observed in AKP organoids (Figures 3I and 3J). LGR5 expression was reduced by random mutations in the DKK2 gene and the knockout clones showed about three-fold reduction in LGR5 expression compared to the control AKP organoids at day 8 (Figures S2G and S2H). Also, elevation of differentiation marker genes such as Emp1 and Slc26a3 was confirmed (Figure 3I). Decreased spheroid/enteroid ratios in morphology reflect reduced stemness in KO organoids as well (Rodriguez-Colman et al., 2017) (Figures 3J and S4A). These data confirmed the necessity of DKK2 in the stemness of colorectal cancer represented by enhanced LGR5 expression and the underlying mechanisms may not be through the canonical Wnt signaling pathway.

DKK2-driven loss of HNF4a×1 protein enhances LGR5 expression in colon cancer cells

Ingenuity pathway analysis (IPA) of the RNA-seq data from colitis-induced polyps suggested a transcription factor, hepatocyte nuclear factor 4-alpha (HNF4A) as an upstream regulator that is activated in the absence of DKK2 (Figure S5A). HNF4A is a nuclear receptor protein which is crucial for development of the gut (Chen et al., 2019). VillinCremERT2-Hnf4afl/fl conditional knockout mice showed destabilization of intestinal epithelium following increased expression of LGR5 upon tamoxifen administration (Cattin et al., 2009). Chellappa et al. have reported the existence of two Hnf4a isoforms—Hnf4a1 and Hnf4a7—in the gut epithelium and have discovered the loss of the HNF4a1 isoform in AOM/DSS-driven intestinal tumor cells (Chellappa et al., 2016). Furthermore, inhibition of the HNF4a1 isoform using shRNA in the Caco2/15 human colon cell line showed reduced ileum villi cells signature and colon differentiated cell signature by gene set enrichment analysis (Babeu et al., 2018). Briefly, loss of HNF4A in colorectal cancer impairs epithelial differentiation.

We tested whether HNF4A is involved in DKK2 downstream signaling in colorectal cancer using AKP and KO organoids. Quantitative gene expression analysis showed that two isoforms of HNF4A, HNF4a1 and HNF4a7, were unchanged in the absence of DKK2 (Figure S5B). On the other hand, the protein level of HNF4a1 was...
Flow cytometry analysis of LGR5 expression and c-Src phosphorylation (pSrc) in HNF4

Flow cytometry analysis of c-Src phosphorylation (pSrc) and LGR5 expression in AKP organoids after 5

Quantitative gene expression analysis of LGR5 and DKK2 in dasatinib treated AKP organoids at 24 hr in a dose-dependent manner.

Statistic analysis of the percent of cells expressing high levels of LGR5 (LGR5-high: LGR5 and pSrc double-positive) and the mean fluorescence intensity of pSrc in (E).

Flow cytometry analysis of c-Src phosphorylation (pSrc) and LGR5 expression in KO organoids.

Quantification of HNF4

Statistical analysis of the percent of LGR5-high cells and the mean fluorescence intensity of pSrc in HNF4α1 KD.

Confocal microscopy of HNF4α1 staining in AKP organoids carrying the tdTomato reporter gene in the presence or absence of 5 μM of c-Src inhibitor, dasatinib for 48 hr. Scale bar = 20 μm.

Statistial analysis of HNF4α1 positive cells per high power field (HPF) in (K). Each symbol represents a single image of HNF4α1 staining.

Quantitative gene expression analysis of LGR5 in HNF4α1 KD organoids.

Flow cytometry analysis of LGR5 expression and c-Src phosphorylation (pSrc) in HNF4α1 KD.

Confocal microscopy of HNF4α1 staining in AKP organoids carrying the tdTomato reporter gene. Scale bar = 1 μm.

Quantitative analysis of pSrc double-positive cells in (A) using ImageJ. Each dot represents a single image of HNF4α1 staining.

Flow cytometry analysis of c-Src phosphorylation (pSrc) and LGR5 expression in KO organoids.

Statistical analysis of the percent of cells expressing high levels of LGR5 (LGR5-high: LGR5 and pSrc double-positive) and the mean fluorescence intensity of pSrc in (E).

Quantification of HNF4α1 protein in the HNF4α1 knockout in DKK2 KO AKP organoids (HNF4α1 KD) by Western blot analysis.

Quantitative gene expression analysis of LGR5 in HNF4α1 KD organoids.

Flow cytometry analysis of LGR5 expression and c-Src phosphorylation (pSrc) in KO organoids.

Statistical analysis of the percent of LGR5-high cells and the mean fluorescence intensity of pSrc in HNF4α1 KD.

Confocal microscopy of HNF4α1 staining in AKP organoids carrying the tdTomato reporter gene. Scale bar = 1 μm.

Statistical analysis of HNF4α1 positive cells per high power field (HPF) in (K). Each symbol represents a single image of HNF4α1 staining.

Quantitative gene expression analysis of LGR5 and DKK2 in dasatinib treated AKP organoids at 24 hr in a dose-dependent manner.

Flow cytometry analysis of c-Src phosphorylation (pSrc) and LGR5 expression in AKP organoids.

Quantification of HNF4α1 knockdown in DKK2 KO AKP organoids (HNF4α1 KD) by Western blot analysis.

Flows cytometry analysis of c-Src phosphorylation (pSrc) and LGR5 expression in DKK2 knockdown (Figures 4I and 4J). Those data confirmed that protein depletion of HNF4α1 by c-Src is a key step in DKK2 downstream signaling for LGR5 expression.

To validate the inhibitory effects of HNF4α1 in LGR5 expression in colon cancer, we performed a loss of function study targeting the HNF4α1 gene in KO organoids where the protein level of HNF4α1 was increased by DKK2 knockout (Figure 4G). Transcriptional expression of LGR5 was recovered by CRISPR-induced random loss of function mutations in the HNF4α1 gene in KO organoids (Figure 4H).

The protein level of c-Src in AKP organoids was demonstrated by Western blotting after 24 hr of dasatinib treatment.

The percentile of cells expressing high levels of LGR5 in KO organoids was also increased by HNF4α1 knockdown (Figures 4I and 4J). Those data confirmed that protein depletion of HNF4α1 by c-Src is a key step in DKK2 downstream signaling for LGR5 expression.

c-Src-induced HNF4α1 phosphorylation has been reported as an underlying mechanism of its degradation in human colorectal cancer cells (Chellappa et al., 2012b). It has been reported that activation of c-Src is sufficient to drive stem cell expansion in intestinal tumorigenesis (Cordero et al., 2014). To validate whether c-Src is the downstream target of DKK2 in the process of HNF4α1 protein degradation, we utilized a well-known c-Src inhibitor, dasatinib in AKP organoid culture. Inhibition of c-Src activation using dasatinib showed increased HNF4α1 protein in the nucleus of AKP organoids similar to what has been shown in KO organoids (Figures 4K and 4L). Reduced transcription of LGR5 and DKK2 was observed in dasatinib-treated AKP organoids in a dose-dependent manner (Figure 4M). Flow cytometry analysis of c-Src phosphorylation and LGR5 expression further confirmed that c-Src activation is critical to elevate the number of cells expressing high levels of LGR5 in AKP organoids (Figures 4N and 4O). Following the loss of function study using dasatinib, we performed a gain of function study using recombinant DKK2 protein and normal colon organoids, which have marginal expression of DKK2. Recombinant DKK2 protein treatment in normal colon organoid culture increased c-Src phosphorylation about two-fold compared to untreated controls (Figures S6A and S6B). The percentile of LGR5 positive cells in the recombinant DKK2-treated organoids was increased about two-fold as well (Figure S6C). Taken together, our findings suggest DKK2 as an essential factor for the stemness of colorectal cancer represented by enhanced LGR5 expression.

**DISCUSSION**

Increased expression of the intestinal stem cell marker gene LGR5 is commonly observed in colorectal cancers (Leushacke and Barker, 2012; Szakandera et al., 2015; Li et al., 2016). Recent studies have shown that depletion of
LGR5 expressing cells suppressed metastatic progression of colon cancer in murine model systems (de Sousa e Melo et al., 2017; Fumagalli et al., 2020). Here, we report that DKK2 is required for enhanced LGR5 expression in colorectal cancer. We have confirmed an increased expression of DKK2 in colorectal cancer and identified its positive feedback regulation. DKK2 facilitates c-Src activation followed by proteasomal degradation of the transcription factor HNF4α1. Loss of HNF4α1 elevated LGR5-expressing cancer stem cell populations. These alterations by DKK2 eventually promote colorectal cancer development.

DKK2 is known as a Wnt antagonist in the presence of the receptor Kremen-2 (Wu et al., 2000). Marginal expression of Kremen-2 in colon cancer cells potentiates the agonistic roles of DKK2 in the gut epithelium (Uhlén et al., 2015). Abnormal proliferation of the gut epithelium is often caused by alterations in the β-catenin-induced Wnt canonical pathway (Shtutman et al., 1999). However, proliferation of cancer cells was unchanged in the absence of DKK2 in colitis-induced polyps and colon cancer organoids. We think this is the consequence of functional loss of APC in both colitis-induced tumors and our APC knockout cancer organoids (Filippo et al., 1998). It permanently activates β-catenin such that the Wnt agonistic or antagonistic functions of DKK2 are minimized in this context. Ingenuity pathway analysis of our RNA-seq data also showed marginal reduction of Wnt/β-catenin signaling in the DKK2 knockout polyps whereas basal carcinoma signaling was significantly reduced. Expression of the canonical Wnt target genes was unaltered by DKK2 knockout in APC knockou cancer organoids. Cellular localization and the protein quantity of β-catenin in cancer organoids were unaffected by DKK2 knockout as well. Collectively, DKK2 effects on the Wnt canonical signaling pathway were not apparent in colon epithelial cells in the context of functional loss of APC such as colitis-induced tumors.

We showed that DKK2 increased LGR5 expression in colon cancer cells. In the homeostatic condition, intestinal stem cells are primed with Wnt ligands such as Wnt3a for LGR5 expression (Clevers, 2013). In colorectal cancer, a recent study has shown that a transcription factor, GATA6 increases LGR5 expression through repressing BMP4 signaling (Whissell et al., 2014). GATA6 directly bound to the LGR5 promoter in this report. DKK2 knockout in AKP organoids increased BMP4 transcription whereas GATA6 expression was unchanged (Figure S5E). Future studies could investigate the molecular mechanisms of DKK2 and HNF4α1 in BMP4-mediated or direct regulation of LGR5 expression in colorectal cancer.

c-Src activation is commonly observed in aggressive colon cancers and is involved in cell adhesion, invasion, and migration of cancer cells which are essential for metastasis (Lieu and Kopetz, 2010; Cordero et al., 2014; Chen et al., 2014). The underlying mechanisms of c-Src activation in colon cancer cells are still unclear. Our results showed significant reduction of c-Src phosphorylation at Y416 in DKK2 knockout colon cancer organoids even in the presence of APC, KRAS, and TP53 mutations. Gain of function study using recombinant DKK2 further confirmed that DKK2 activates c-Src in normal colonic epithelial cells as well. These findings propose DKK2 as a key upstream regulator of c-Src activation in colonic epithelial cells during tumorigenesis. Further studies to identify the DKK2 receptors in c-Src activation will provide better molecular understanding of the DKK2 downstream signaling pathways.

In this study, we have shown that DKK2 is required for the elevated expression of LGR5 in colon cancer cells through c-Src activation and depletion of HNF4α1. Based on our findings, new therapeutic approaches targeting DKK2 would be beneficial against colorectal cancers, particularly to control LGR5 expressing cancer stem cells.

Limitations of the study
Increased expression of DKK2 is shown in the colitis-induced tumor model and cancer organoids with mutations in APC, KRAS, TP53, and SMAD4 genes. Based on the pattern of DKK2 expression, this study focused on the roles of DKK2 in colon cancer cells. Further study is needed to understand the roles of DKK2 in the homeostatic condition. In this study, we observed decreased expression of LGR5 by DKK2 knockout in colitis-induced polyps and cancer organoids. LGR5 is expressed in most stem cells including crypt base columnar stem cells and quiescent stem cells and paneth cells as well. A technical limitation of our data is that we cannot provide information about the sub-types of LGR5 expressing stem cells affected by DKK2 knockout.

Resource availability
Lead contact
Further requests for resources should be directed to and will be fulfilled by the lead contact, Alfred L. M. Bothwell (alfred.bothwell@yale.edu).
Materials availability
This study did not generate new materials.

Data and code availability
The accession number for the RNA sequencing data reported in this study is GSE157535.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102411.

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AUTHOR CONTRIBUTIONS
J.S. and A.L.M.B. designed and conducted the experiments, analyzed the data and wrote the manuscript. J.J. performed immunofluorescence and confocal microscopy and Western blot analysis. J.C. and J.L. performed and analyzed RNA-seq experiments. R.K.D. cloned gRNA/Cas9 plasmids targeting DKK2 exon1 and HNF4a exon1. J.B. and O.Y. developed colon tumor organoids. J.H. contributed to RNA-seq and quantitative PCR analysis. S.E.M. supported in vivo experiments. M.C.A.V. was involved in colonoscopy. W.K., J.K., and J.C. generated recombinant mouse DKK2 protein and polyclonal anti-DKK2 antibody. W.T. and D.W. developed DKK2-floxed mice. H.N.B., R.X.M. and X.L. contributed to immunohistochemistry. J.S. performed all other experiments.

DECLARATION OF INTERESTS
The authors declare no competing interests exist.

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Supplemental information

Dickkopf-2 regulates the stem cell marker LGR5 in colorectal cancer via HNF4α1

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Supplemental figures and legends

Figure S1. Lgr5 expression and Dkk2 knockout phenotype in the colitis-induced tumor model using VillinCre-Dkk2ffl and tamoxifen-inducible Lgr5CreERT2-Dkk2ffl knockout mice (Related to Figures 1 and 2). (A) Sorting strategy for tumor-infiltrating lymphocytes (CD45posEpCAMneg), differentiated epithelial cells (CD45negEpCAMposLGR5-EGFPneg), Paneth-like cells and transit amplifying cell (CD45negEpCAMposLGR5-EGFPmid) and stem cells (CD45negEpCAMposLGR5-EGFPhigh) in colitis-induced tumors. Each population was sorted from the 7AAD negative live cell population. (B) Quantitative Lgr5 expression analysis in the sorted cells. (C) Quantitative gene expression analysis of Dkk2 in tumor tissues from AOM/DSS-treated VillinCre-Dkk2ffl knockout mice. WT N: wild type normal colon, n=4, WT T: wild type tumor, n=16, KO N: Dkk2 knockout normal colon, n=4, KO T: Dkk2 knockout tumor, n=20. (D and E) Proliferation analysis of AOM/DSS-induced tumors by Ki-67 staining (D) and statistical analysis (E). (F) Expressional correlation of Dkk2 and Lgr5 in collected tumor tissues in WT and tamoxifen-inducible KO mice. Each dot represents an individual tumor tissue. Pearson r test. (G)
Tumor number and score in $Lgr5^{\text{CreERT2-IRES-EGFP}}$ mice ($Lgr5$, $n=7$) having loss of one $Lgr5$ allele by insertion of the $Lgr5^{\text{CreERT2-IRES-EGFP}}$ transgene compared to wild type C57BL/6 (WT, $n=11$) mice after AOM/DSS treatment. n.s, not significant, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, error bars represent ± s.d.; two-tailed Welch’s t-test. $n$ represents the number of independent animals.
**Figure S2.** Generation of *Dkk2* knockout colon cancer organoids. (A) Quantitative PCR analysis of *Lgr5* expression in colonic organoids carrying various mutations. Wild type organoids were cultured in the WERN medium containing Wnt3a, EGF, Rspandin1 and Noggin. *Apc* only mutant organoids were grown in the EGF only containing medium. *Apc, Kras, Tp53* and *Smad4* double, triple and quadruple mutant organoids culture used the minimal
medium without all of those four growth factors. WT; wild type normal colonic organoids, A; Apcc
knockout organoids, AK; Apcc knockout-KrasG12D mutant organoids, AKP; Apcc knockout-KrasG12D-
Tp53 knockout mutant organoids. AKPS; Apcc knockout-KrasG12D-Tp53 knockout-Smad4 knockout
mutant organoids. (B-C) Expression of Dkk2 and Lgr5 in AKP triple mutant organoids was
analyzed in a time-dependent manner. (D) A schematic diagram of the development of Dkk2
knockout AKP organoids. Isolated AKP organoid cells were transduced with lentivirus carrying
Dkk2 guide RNA, Cas9 and Puromycin resistant genes. Transduced Dkk2 knockdown (KD) cells
were selected in the presence of Puromycin. The selected cells were sorted as a single cell then
cloned. Each Dkk2 knockout (KO) clone was identified by sequencing genomic DNA. (E)
Sequence of guide RNA targeting Dkk2 exon1. Representative genomic DNA sequence and
amino-acid sequence of DKK2 in the knockout AKP organoids. KO clone #1 is shown. Frame
shift mutation in Dkk2 exon1 resulted in a truncated DKK2 protein (Red box; PAM sequence. G
was omitted. 1-33 S.P; signal peptide. 34-56; truncated 23 amino acids, HLQA-Stop; newly
generated sequence by frame shift mutation). Normal and truncated DKK2 protein structures are
displayed. (F-H) Organoids were dispersed as single or 2-3 attached cells by TrypLE enzyme
digestion. The isolated cells were suspended in matrigel. Once matrigel solidified, the AKP
organoid culture medium was added. The culture medium was replaced every other day.
Quantitative PCR analysis of Dkk2 and Lgr5 expression in KO organoids at day 8 after plating
isolated single organoid cells (F and G). 3 independent KO clones are shown for Lgr5 expression
(H). WT; wild type colonic organoids cultured in the WERN medium, AKP; Untreated AKP
organoids, Scrambled; Scrambled guide RNA transduced AKP organoids, KO; unsorted
douclonal Dkk2 knockout organoids, KO with #; sorted Dkk2 knockout organoid clones. n.s, not
significant, *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001; two-tailed Welch’s t-test. Error bars
indicate mean ± s.d. Results are representative of two identical independent experiments.
Figure S3. Analysis of CTNNB1 protein in Dkk2 knockout AKP organoids (Related to Figure 3). (A) Confocal microscopy analysis of CTNNB1 in control (AKP) and Dkk2 knockout (KO) AKP organoids. Scale bar = 5 μm. (B) Quantification of CTNNB1 protein in control (red) and Dkk2 knockout (blue) AKP organoid cells at day 3 after plating isolated single organoid cells by intracellular staining flow cytometry analysis. Results are representative of at least three biological replicates.
Figure S4. Morphology of AKP organoids (Related to Figure 3). (A) Images were captured at the indicated time points after plating the isolated cells for 10 days. Control AKP organoids formed multi-circular morphology whereas Dkk2 knockout AKP organoids showed linearized and entangled morphology. Scale bar = 5 µm.
Figure S5. mRNA and protein levels of HNF4α1 in Dkk2 knockout AKP organoids (Related to Figure 4). (A) Ingenuity pathway analysis suggested upstream regulators in the absence of Dkk2 from AOM/DSS-treated colitis induced polyps. (B and E) Quantitative gene expression analyses of Hnf4a isoforms (B) and Bmp4 and Gata6 (E) on day 8 after passaging control (AKP) or Dkk2 knockout AKP (KO) organoids. (C and D) Confocal microscopy of HNF4α1 protein in AKP organoids. Statistical analysis of HNF4α1 positive cells in 3 independent Dkk2 knockout (KO) AKP organoid clones (C). n.s = not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; two tailed Welch’s t test. Error bars represent ± s.d. HPF, high power field.

Scrambled, scrambled guide RNA transduction. Numbers (#1-3) indicate the clones. Each symbol represents a single image of HNF4α1 staining. Results are representative of two identical independent experiments. Representative images of HNF4α1 staining in wild type and Dkk2
knockout AKP organoids with higher magnification (D). Green; HNF4α1, Red; tdTomato, Blue; DAPI, Scale bar = 2 µm.

**Figure S6.** DKK2-induced phosphorylation of c-Src in normal colonic organoids (Related to Figure 4).

(A) Flow cytometry analysis of phosphorylated c-Src (pSrc) and LGR5 expression in normal colonic organoids in the presence of 1 µg/ml of recombinant mouse DKK2 (rmDKK2) protein for 12 h. (B) Statistic analysis of the mean fluorescence intensity (MFI) of pSrc in (A). (C) Statistic analysis of the percentile of LGR5 positive stem cells in normal colonic organoids in (A).

****P<0.0001; two-tailed Welch’s t-test. Error bars indicate mean ± s.d. Results are representative of three independent experiments. Each symbol represents an independent sample in each group.

**Table S1.** The list of quantitative PCR primers (Related to Figures 1-4).

| Gene | F                        | R                          |
|------|--------------------------|-----------------------------|
| Bmi1 | ATCCCCACTTAATGTGTGTCCT   | CTTGCTGGTCTCCAAGTAACG       |
| BMP4 | TTCCTGGTAAACGAATGCTGA    | CCTGAATCTCGGCAGCTTTTT       |
| Gene   | F               | R               |
|--------|-----------------|-----------------|
| DKK2   | GTACCGCGCTGCAATAATGGAAATCT | ATCCCAAGGTCATGGTTTGGAATAGT |
| Emp1   | TGTGTGCTACTGGCTGGTCT | CATGCCGCTAGGACAGGGAG |
| FoxM1  | CTGATTCTCAAAGACGGAGGC | TTAGTAATCTTGATTCGCTGG |
| GATA6  | TTGCTCGGTAACACAGCAGTG | GTGGTCTGGTGTAGAAGGA |
| HNF4α1 | ATGCAGCTCTCTAAACCTTTG | ACCTTCAGATGGGGACGTG |
| HNF4α7 | TTCTATGCCCCTACAGGGT | TGTCCTATGGGGGTACACA |
| HPRT   | CTCCCCAGACCGCTTTTGTG | TCATCGCTAATCACGACGCT |
| mKi67  | ATCATGGCGCTCCTTTAGGT | GCTCGCCTGGATGGTTCCT |
| Krt20  | AGTTTTCCACGGAACTGCTGTTTC | GTAGCTCATTACGGCTTGGAG |
| LEF1   | TGTTATCCCATACGGGTGG | CATGGAAGTGTCCCTGACAG |
| Lgr5   | GGGAGCGTTCACGGGCCTTC | GGTTGCCATCTAGGCGCAGGG |
| Myc    | CCCTATTTCAATCTCGGACGAG | GAGAAGGACGTAGCGACCG |
| RNF43  | CCGGTCATTTCGGTGCTTC | CCTGGTCTCGGAAGATGGAG |
| Slc26a3| GCCGTTGGTGGGGAACATGA |
| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-DKK2 antibody (polyclonal) | Millipore Sigma | Cat# 06-1087, RRID:AB_10806082 |
| Rabbit normal IgG control antibody | Millipore Sigma | Cat# 12-370, RRID:AB_145841 |
| Ki-67 Monoclonal Antibody (Clone SolA15) | Thermo Fisher Scientific | Cat# 14-5698-82, RRID:AB_10854564 |
| Anti-phospho-Src (Ty416) antibody (Clone 9A6) | Millipore Sigma | Cat# 05-677, RRID:AB_309898 |
| Mouse IgG3 Isotype control | Thermo Fisher Scientific | Cat# 14-470120 |
| BV421 Rat anti-mouse IgG3 antibody (Clone R40-82) | BD Biosciences | Cat# 565808, RRID:AB_2739364 |
| Anti-HNF4a1 antibody (Clone K9218) | R&D Systems | Cat# PP-K9218-00, RRID:AB_2116902 |
| Mouse IgG2A Isotype control | R&D Systems | Cat# MAB003, RRID:AB_357345 |
| Goat anti-mouse IgG2a secondary antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-21131, RRID:AB_2535771 |
| LGR5 mouse monoclonal antibody (Clone 2A2 Dylight488 conjugated) | OriGene | Cat# TA400002, RRID:AB_2622395 |
| Mouse IgG1 Isotype Control, Dylight488 conjugate antibody | Thermo Fisher Scientific | Cat# MA1-191-D488, RRID:AB_2536927 |
| PE-Cyanine7 Anti-Mouse CD45 (Clone 30-F11) antibody | Tonbo Biosciences | Cat# 60-0451, RRID:AB_2621848 |
| CD326 (EpCAM) Monoclonal Antibody (Clone G8.8, APC) | Thermo Fisher Scientific | Cat# 17-5791-80, RRID:AB_2734965 |
| CD16/32 Monoclonal Antibody (Clone 93) | Thermo Fisher Scientific | Cat# 14-0161-82, RRID:AB_467133 |

### Chemicals, Peptides and Recombinant Proteins

| REAGENT OR RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Azoxy methane | Sigma-Aldrich | Cat# A5486 |
| Item                                                                 | Supplier              | Catalog Number       |
|----------------------------------------------------------------------|-----------------------|----------------------|
| DEXTRAN SULFATE SODIUM SALT (36,000-50,000 M.Wt.) MP Grade           | MP Biomedicals        | Cat# 0216011080      |
| Tamoxifen                                                            | Sigma-Aldrich         | Cat# T5648           |
| Sunflower seed oil from Helianthus annuus                           | Sigma-Aldrich         | Cat# S5007           |
| Recombinant mouse DKK2 protein                                      | R&D Systems           | Cat# 2435-DKB-010     |
| Puromycin                                                            | Sigma-Aldrich         | Cat# P9620           |
| Blasticidin                                                          | Sigma-Aldrich         | Cat# 15205           |
| Dasatinib                                                            | Sigma-Aldrich         | Cat# CDS023389       |
| Recombinant murine EGF                                              | Peprotech             | Cat# 315-09          |
| Nutlin-3                                                             | Cayman                | Cat# 10004372        |
| PEG-itTM Virus Precipitation Solution                               | SBI                   | Cat# LV810A-1        |
| Hexadimethrine bromide (Polybrene)                                  | Sigma-Aldrich         | Cat# H9268           |
| Y-27632                                                              | Tocris                | Cat# 1254            |
| B-27® Supplement (50X), minus vitamin A                              | Thermo Scientific     | Cat# 12587010        |
| N-2 Supplement (100X)                                                | Thermo Scientific     | Cat# 17502048        |
| Advanced DMEM/F-12                                                   | Thermo Scientific     | Cat# 12634010        |
| TrypLE™ Express Enzyme (1X)                                          | Gibco                 | Cat# 12605010        |
| Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free | Corning              | Cat# 356231          |
| GlutaMAX™ Supplement                                                 | Gibco                 | Cat# 35050061        |
| Collagenase IV                                                       | Worthington Biochemical | Cat# LS004188     |
| Dispase II, protease                                                 | Sigma-Aldrich         | Cat# D4693           |
| 16% Paraformaldehyde (PFA) solution, EM grade                       | Thermo Scientific     | Cat# 50-980-488      |
| Normal Goat Serum (10%)                                              | KPL                   | Cat# 71-00-27        |
| Glycogen, RNA grade                                                  | Thermo Scientific     | Cat# R0551           |
| TRIzol Reagent                                                       | Thermo Scientific     | Cat# 15596018        |
| Zombie Aqua™ Fixable Viability Kit                                   | Biolegend             | Cat# 423102          |

**Critical Commercial Assays**

| Item                                                                 | Supplier              | Catalog Number       |
|----------------------------------------------------------------------|-----------------------|----------------------|
| Foxp3 / Transcription Factor Staining Buffer Set                     | Thermo Fisher Scientific | Cat#00-5523-00       |
| RNaseasy kit                                                         | QIAGEN                | Cat#74106            |
| miRNeasy kit                                                        | QIAGEN                | Cat#217004           |
| Illumina TruSeq stranded mRNA preparation kits                       | Illumina              | Cat#20020594/20020595 |
| IntestiCultTM Organoid Growth Medium (Mouse)                         | Stemcell              | Cat# 06005           |
| DAB Peroxidase HRP Substrate Kit (with Nickel), 3,3'-diaminobenzidine | VECTOR Laboratories   | Cat# SK-4100         |
| R.T.U. elite ABC Reagent                                            | VECTOR Laboratories   | Cat# PK-7100          |
| ProLong™ Gold Antifade Mountant with DAPI                            | Thermo Fisher Scientific | Cat# P36941          |
| SuperSignal™ West Femto Maximum Sensitivity Substrate                | Thermo Fisher Scientific | Cat# 34904           |
Experimental Models: Organisms/Strains

| Mouse: C57BL/6J | The Jackson Laboratory | JAX:000664 |
|-----------------|-------------------------|------------|
| Mouse: Villin<sup>Cre+/-</sup>-DKK2<sup>fl/fl</sup> | This paper | N/A |
| Mouse: Lgr5<sup>CreERT2-IRES-EGFP</sup>-DKK2<sup>fl/fl</sup> | This paper | N/A |

Sequence-Based Reagents

| Primers for qPCR, A table attached below | This paper | N/A |
|-----------------------------------------|------------|------|
| sgRNA for CRISPR gene editing, A table attached below | This paper | N/A |

Software and Algorithms

| FlowJo v10.5 | Tree Star | RRID:SCR_008520 |
|-------------|-----------|-----------------|
| GraphPad Prism 7.0 | GraphPad Software | RRID:SCR_002798 |
| ZEN Digital Imaging Light Microscopy | Zeiss | RRID:SCR_013672 |
| Image J | NIH | RRID:SCR_003070 |
| Bruker MS FX PRO software | Bruker | RRID:SCR_017365 |
| IPA 48207413 | QIAGEN | RRID:SCR_008653 |
| TopHat v2.1.0 | Johns Hopkins University CCB | RRID:SCR_013035 |
| HTSeq v0.8.0 | HTSeq | RRID:SCR_005514 |
| DESeq v2 | Bioconductor | RRID:SCR_000154 |

Recombinant DNA

| LentiCRISPR v2 | Addgene | Cat# 52961 |
|----------------|---------|------------|
| LentiCRISPR v2-Blast | Addgene | Cat# 83480 |

Other

| 8-well Chamber Slide w/ removable wells | Thermo Fisher Scientific | Cat# 154534PK |
|----------------------------------------|--------------------------|---------------|
| Lysing Matrix D, 2 mL Tube | MP Biomedicals | Cat# 116913-100 |

Animals

All studies handling animals were approved by institutional animal care and use committee at the Yale University School of Medicine animal facility. Dkk2<sup>fl/fl</sup> mice were developed by following procedures. A targeting vector was designed to insert a loxP site upstream of Exon 2 of the Dkk2 gene, followed by a frt-flanked neomycin resistance (neo) cassette. An additional loxP site was added downstream of Exon 3. The construct was electroporated into 129 embryonic stem (ES) cells, and correctly targeted ES cells were injected into blastocysts. Chimeric males were bred with C57BL/6 females, and their offspring were bred with ROSA26-Flpe mice to remove the PGKneo cassette. Resulting Dkk2-floxed mice were crossed to remove the Flp-expressing allele, and subsequently backcrossed to C57BL/6N mice for more than 5 generations. Lgr5<sup>CreERT2-IRES-</sup>
EGFP mice were gifted from Dr. Richard Flavell. Villin^{Cre} mice were gifted from Dr. Ruslan Medzhitov. The Villin^{Cre} and Lgr5^{CreERT2-IRES-EGFP} mice were crossed with Dkk2^{fl/fl} mice to generate conditional Dkk2 knockout mice.

**Colitis-induced tumor model**

12.5 mg/kg of azoxymethane (AOM, Sigma, A5486) was administered to 6-8 weeks old Villin^{Cre/+}-Dkk2^{fl/fl} or Lgr5^{CreERT2-IRES-EGFP/+}-Dkk2^{fl/fl} mice via intra-peritoneal injection. Villin^{+/-}-Dkk2^{fl/fl} or Lgr5^{+/-}-Dkk2^{fl/fl} littermate mice were used as controls. Both male and female mice were subjected and the number of gender was matched in experimental and control groups. After 5 days, mice were treated with 2.5 % of dextran sodium sulfate (DSS, MP biomedicals, molecular mass 36,000–50,000 Da) in the drinking water for 5 days followed by 14 days of regular water. This cycle was performed three times. 7 days after DSS treatment in each cycle, 200 µl of tamoxifen (10 mg/ml in sunflower oil, Sigma, T5648) was delivered into Lgr5^{CreERT2-IRES-EGFP/+}-Dkk2^{fl/fl} mice and their littermate controls by intra-peritoneal injection every other day, total three injections per DSS cycle.

**Endoscopy**

Colonoscopy was performed using the Coloview system (Karl Storz) following the manufacturer’s protocol. Mice were anaesthetized by isoflurane. Total number and sizes of tumors were recorded during colonoscopy in a blinded fashion. Tumor sizes were scored from 0 to 5. Sum of all tumor sizes in a single mouse were described as the tumor score.

**RNA isolation**

Total RNA of normal colon tissues, AOM/DSS-induced polyps, normal or tumor colonic organoids were extracted using Trizol reagent (Thermo, 15596018). Tissues and tumors were homogenized with lysing matrix D (MP bio, 116913) in 1 ml of Trizol. Organoids in Matrigel culture were mechanically disrupted and spun at 400 g and 4°C for 5 min. Pellets were re-suspended with PBS and this was repeated twice, then pellets were dissolved in Trizol. RNA cleanup was
performed using miRNeasy kit (Qiagen, 217004) with on-column DNase digestion (Qiagen, 79254) according to the manufacturer’s instructions.

**RNA-seq analysis**

Total RNA was extracted from the AOM/DSS-induced polyps from wild type and Lgr5\(^{CreERT2}\)\(\text{-}\)Dkk2\(^{fl/fl}\) mice or colon cancer organoids. RNA-seq libraries were constructed following Illumina TruSeq Stranded mRNA protocol (20020594). The RNA-seq libraries were sequenced on the Illumina HiSeq 2500 instrument platform (76 bp single-end sequencing) or Illumina Nextseq 500 (42 bp paired-end run). The sequencing reads were aligned onto Mus musculus GRCm38/mm10 reference genome using the TopHat v2.1.0 software or Kallisto v0.45.0. The mapped reads were transformed into the count matrix with default parameters using the HTSeq v0.8.0 software, then normalized using the DESeq v2 software. Differentially expressed genes (DEGs) were identified using the same software based on a negative binomial generalized linear model. Sleuth was used to analyze statistical significance of kallisto data.

**Quantitative real time PCR**

1-5 \(\mu\)g of total RNA was used for cDNA synthesis using the SMARTer cDNA synthesis kit (Clontech, 634925) following the manufacturer’s protocol. Quantitative real time PCR was performed in 10 \(\mu\)l reactions using the iTag universal SYBR Green supermix on the CFX96 Touch™ real-time PCR detection system. PCR primers are listed in the supplementary table 1.

**Organoid culture**

Colonic epithelium was isolated as previously described (Andersson-Rolf et al., 2014). The isolated colonic crypts were dissolved in 50 \(\mu\)l of 1:1 ratio mixed Matrigel (Corning, 356231) and the IntestiCult™ organoid growth medium, STMECELL, 06005), then plated in a 24 well plate. Once Matrigel was solidified, organoids were incubated in 500 \(\mu\)l of the organoid growth medium. The organoid medium was replaced every other day. Recombinant mouse DKK2 protein (R&D, 2435-DKB-010) was added in a dose- and time-dependent manner. Colon tumor organoids
carrying various combinations of mutations in APC, KRAS, TP53 and SMAD4 and the tandem dimer Tomato (tdTomato) reporter gene were generated as described below. The minimal medium containing 1x B27 supplement (Thermo, 12587010), 1x Glutamax (Gibco, 35050061) and 1x Penicillin/Streptomycin (Thermo, 15140122) in advanced DMEM-F12 (Thermo, 12634010) was prepared. Those tumor organoids were plated in 1:1 ratio of Matrigel and the minimal medium mix. APC only (A), APC and TP53 double mutant (AP) organoids were cultured in the minimal medium with 50 ng/ml of EGF. APC and KRAS double mutant (AK), APC, KRAS and TP53 triple mutant (AKP) and APC, KRAS, TP53 and SMAD4 quadruple mutant organoids were cultured in the minimal medium. Culture medium was replaced every other day.

**Development of colon tumor organoids**

Colon crypts were isolated from

[1]: *Rosa-LSL-tdTomato* mice,

[2]: *LSL-Kras^{G12D/+}* *Rosa-LSL-tdTomato* mice,

[3]: *LSL-Kras^{G12D/+}Tp53^{fl/fl}* *Rosa-LSL-tdTomato* mice.

Organoid lines were cultured in the WRN media with recombinant mouse EGF (50 ng/ml, Peprotech 315-09). Then, organoids were transfected with *pSECC-Apc* plasmid (containing Cre, Cas9 and sgApc) to generate

[1] *Apc^-/+* *tdTomato*^+, 

[2] *Apc^-/Kras^{G12D/+}* *tdTomato*^+, 

[3] *Apc^-/Kras^{G12D/+}Tp53^-/+* *tdTomato*^+ organoids.

*Apc* deletion was selected by Wnt3A withdrawal, *Tp53^-/+* was selected by Nutlin-3 addition (10 µM) and *Kras^{G12D}* was selected by EGF withdrawal. After establishment of the *Apc^-/-* 

*Kras^{G12D/+}Tp53^-/+* *tdTomato*^-+* organoid line, subsequent transfection with *pSECC-Smad4* plasmid (containing Cre, Cas9 and sgSmad4) was performed. *Smad4* deletion was selected by addition of TGFβ (10 ng/ml) to the media to generate

[4] *Apc^-/Kras^{G12D/+}Tp53^-/+Smad4^-/+* *tdTomato*^-+* organoids.
Design and cloning gRNA/Cas9 plasmids targeting Dkk2 and Hnf4α1

sgRNAs of Dkk2 and Hnf4α1 were cloned into LentiCRISPRv2 or LentiCRISPRv2-blast respectively according to an established protocol (Ran et al., 2013). LentiCRISPRv2 (plasmid #52961) and LentiCRISPRv2-blast (plasmid #83480) were purchased from the Addgene (Sanjana et al., 2014). sgRNAs targeting the first exon of mouse Dkk2 and Hnf4α1 were designed using the Broad Institute’s online sgRNA designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), (Doench et al., 2016). Non-targeting control gRNAs were selected from the mouse GeCKO v2 library(Sanjana et al., 2014). Dkk2; 5’- GTTCATGCCTGCAGACGGT TGG -3’, Hnf4α1; 5’-GTAGTCGGCCATATGC -3’, non-targeting sgRNA; GACTCCGGGTACTAAATGTC

Lentiviral transduction

293FT cells were transfected with psPAX2, pMD2.G and LentiCRISPRv2 carrying guide RNA targeting Dkk2 exon1 (5’-GTTCATGCCTGCAGACGGT TGG-3’) using jetPEI (Polyplus, 101-10N) according to the manufacturer’s protocol to generate lentivirus. Lentivirus was precipitated and concentrated by peg-IT (SBI: LV810A-1) solution. AKP organoids were digested as single or 2-3 attached cells in TrypLE solution (Thermo, 12605010) at 37 °C for 10-30 min. Approximately 100,000 isolated cells were mixed with 250 µl of the concentrated lentivirus, polybrene (8 µg/ml) and Y27632 (10 µM, Tocris, 1254) in total 750 µl of advanced DMEM-F12 and plated in a 24 well plate. Spin infection was performed at 600 g, 37 °C for 1 h, then placed in the CO2 incubator for 6 h. The transduced cells were harvested and plated in 50 µl of Matrigel (Corning, 356231). 2-3 days after incubation in the minimal medium (B27, Glutamax and Pen/strep in advanced DMEM-F12), the transduced organoids were selected in the 10 µg/µl of Puromycin containing minimal medium for 7 days. Puromycin was refreshed every other day. Single cell sorting was performed to clone the transduced Dkk2 knockout AKP organoids. Knockout clones have identified by sequencing the guide RNA target region.

To knockdown Hnf4α1 in Dkk2 knockout AKP organoids, we generated lentivirus using LentiCRISPRv2-blast carrying guide RNA targeting Hnf4α1 exon1 (5’-
GTAGTCGGCCATATCCATGC

3') as described above. After lentiviral transduction, Hnf4α1
knockdown organoids were selected in the 10 μg/μl of Blasticidin containing minimal medium for
7 days. The culture medium was refreshed every other day.

Immunostaining and Confocal microscopy
Paraffin-embedded sections were cleared with Xylene and rehydrated with Ethanol and deionized
water using standard methods. Antigen retrieval was performed using the sodium citrate buffer
(10 mM sodium citrate, 0.05 % Tween20, pH 6.0) at 95°C for 30 min. Tissue sections were
stained with anti-DKK2 antibody (Millipore, 06-1087) or anti-Ki67 (Thermo, 14-5698-82) antibody
overnight at 4°C followed and for secondary antibody for 1 hour at room temperature. DAB
staining (Vector Lab) and hematoxylin (Sigma) counter staining were performed for detection
following manufacture’s protocols. Images were acquired with a Qlmaing microscope camera. For
immunofluorescence and confocal microscopy, sections were stained with anti-HNF4α1 antibody
(R&D, pp-k9218), alexa488-conjugated anti-mouse IgG2a secondary antibody, anti-CTNNB1
monoclonal antibody (Invitrogen, 53-2567-42) and DAPI (Sigma, D9542). Pictures were captured
using a Zeiss Axio Observer Z.1 microscope or a Zeiss LSM780 confocal laser-scanning
microscope.

Tumor dissociation
Colitis-induced tumors and liver metastasized tumors were collected and dissociated in the
digestion buffer containing 200 U/ml of type IV collagenase (Worthington, LS004188), 125 μg/ml
of type II dispase (Sigma, D4693), 2.5 % fetal bovine serum, 1x Penicillin/Streptomycin in DMEM
at 37 °C for 30-60 min with agitation. Isolated cells were centrifuged at 400 g and 4 °C for 5 min,
then re-suspended in chilled PBS and filtered through the 40 μm pore size strainer.

Flow cytometry
Single cells isolated from organoids or metastatic tumors were fixed and permeabilized with the
intracellular fixation and permeabilization buffer set (eBioscience, 88-8824-00) following the
manufacturer's instructions. Those cells were stained with DyLight488 conjugated anti-Lgr5 antibody (Clone: OTIA2, Origene, TA400002) or anti-phospho-Src (Ty416) antibody (Clone: 9A6, Milliporesigma, 05-677) and Brilliant violet 421 (BV421) Rat anti-mouse IgG3 antibody (Clone: R40-82, BD Horizon, 565808). Flow cytometry was performed using the Stratedigm-13.

**Supplemental References**
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