Epithelial NOTCH Signaling Rewires the Tumor Microenvironment of Colorectal Cancer to Drive Poor-Prognosis Subtypes and Metastasis

Graphical Abstract

Highlights

- Epithelial NOTCH1 signaling drives metastasis in serrated CRC
- Poor-prognosis CRC subtypes CMS4/CRIS-B are controlled by NOTCH1
- TGF-β-mediated neutrophil infiltration is critical for NOTCH1-driven metastasis
- Neutrophil targeting provides therapeutic opportunity in metastatic CRC

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In Brief

In a genetically engineered mouse model, Jackstadt et al. show that NOTCH1 activation drives metastasis in KRASG12D-driven serrated colorectal cancer (CRC) through TGFβ-dependent neutrophil recruitment. Thus, targeting neutrophil recruitment is a potential therapeutic approach in metastatic CRC.

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Epithelial NOTCH Signaling Rewires the Tumor Microenvironment of Colorectal Cancer to Drive Poor-Prognosis Subtypes and Metastasis

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SUMMARY

The metastatic process of colorectal cancer (CRC) is not fully understood and effective therapies are lacking. We show that activation of NOTCH1 signaling in the murine intestinal epithelium leads to highly penetrant metastasis (100% metastasis; with >80% liver metastases) in KrasG12D-driven serrated cancer. Transcript profiling reveals that epithelial NOTCH1 signaling creates a tumor microenvironment (TME) reminiscent of poorly prognostic human CRC subtypes (CMS4 and CRIS-B), and drives metastasis through transforming growth factor (TGF) β-dependent neutrophil recruitment. Importantly, inhibition of this recruitment with clinically relevant small-molecule inhibitors or therapeutic antibodies has a profound impact on metastatic burden in vivo.

INTRODUCTION

Patient mortality in CRC is closely associated with metastasis (Jemal et al., 2011), with an overall 5-year survival rate for late-stage patients of 5%–10%. Resection of both primary and metastatic lesions provides the best prognosis for these patients, but post-intervention recurrence is very common due to disseminated latent or therapy-resistant tumor cells (Tauriello et al., 2017). Consequently, preclinical models which faithfully recapitulate the processes of CRC metastasis are required.

Significance

CRC is the second most common cause of cancer death, with metastasis the key contributor to CRC-associated death. Thus, a pressing need exists for therapeutic strategies which target disease subtypes with the poorest prognosis. We have generated a genetically engineered mouse model (GEMM) of metastatic CRC, which represents a fundamental advance in preclinical modeling. In doing so, we identify epithelial NOTCH1 signaling as a critical feature of both the poorest prognosis subtypes and of metastatic seeding at the secondary site. Crucially, targeting NOTCH1-driven neutrophil recruitment and TGF-β signaling with clinically relevant small-molecule inhibitors or therapeutic antibodies has a profound impact on metastatic burden in vivo.
Stratification of CRC by transcriptional profiling (Guinney et al., 2015) has allowed classification of CRC into four consensus molecular subtypes (CMSs). CMS1 tumors are likely to be microsatellite unstable, hyper-mutated, and characterized by lymphocytic infiltration. CMS2 and CMS3 tumors exhibit high levels of WNT signaling, little immune infiltration and intermediate overall and relapse-free survival. Patients with “mesenchymal” CMS4 tumors have the worst overall and relapse-free survival rate and these tumors are characterized by significant fibroblast and innate immune cell infiltration, and elevated TGF-β signaling (Dienstmann et al., 2017; Becht et al., 2016a). As these transcriptional signatures are generated from whole tumors, the presence of stromal cells contributes significantly (Calon et al., 2015; Isella et al., 2015; McCorry et al., 2018), confounding analysis. While the contribution of epithelial cells to stromal infiltration/adapta-

tion is not fully understood (Wellenstein and de Visser, 2018), cell-intrinsic transcriptional signatures (CRIS) have been shown to have prognostic implications (Dunne et al., 2017; Isella et al., 2017). In particular, the CRIS-B signature predicts poor prognosis and is enriched for signatures associated with epithelial-mesenchymal transition (EMT) and TGF-β signaling. Notably, while CMS4 and CRIS-B are characterized by the same acti-
vated programs, the composition of gene signatures is different and CRIS-B is a composition of mainly CMS1 and CMS4 genes. Crucially, mutational data does not stratify the different CRC subtypes and the mechanisms which drive subtypes are not known (Guinney et al., 2015).

There are two postulated routes by which metastatic CRC (mCRC) arises. The classical route is initiated by mutations in the APC tumor suppressor gene which is followed by alterations in mitogen-activated protein kinase (MAPK), TP53, and TGF-β signaling during progression (Fearon and Vogelstein, 1999). These tumors develop from adenomas with tubular morphology (Fearon, 2011). Recent efforts to model metastatic disease with combing mutations in the intestine of APC-deficient mice yielded tumors that readily progressed to adenocarcinoma but showed limited metastasis (Sakai et al., 2018). Intriguingly, if APC-deficient tumors with combing mutations are propa-
gated ex vivo as organoids and re-implanted into mice, metas-
tasis occurs (Tauriello et al., 2018; de Sousa e Melo et al., 2017; O’Rourke et al., 2017). Alternatively, CRC progression can be initiated by KRAS or BRAF mutations, with tumor develop-

ment from adenomas with a serrated morphology (Jass et al., 2002). Importantly, patients with serrated adenoma-associated signatures have a poorer prognosis than those with “classical” tubular adenomas (De Sousa et al., 2013). These adenomas may progress to high-grade carcinoma through p16/CDKN2A promoter hyper-methylation and subsequent gene silencing, or via mutation of TP53 (Jusper et al., 2015). Braf-mutant genetically engineered mouse models (GEMMs) of CRC exhibit activated WNT signaling, indicated by nuclear accumulation of β-catenin, while KRAS-mutant tumors appear to develop inde-

pendently of WNT pathway activation (Bennecke et al., 2010; Janssen et al., 2002; Trobridge et al., 2009). Nevertheless, these GEMMs develop few distant metastases and have a long latency.

Despite some caveats, GEMMs are powerful tools to study tu-

mor biology in an autochthonous setting, and are the gold stan-
dard in preclinical CRC research. The major weakness of current CRC GEMMs is the lack of a complete adenoma-carcinoma-metastasis cascade and the absence of highly penetrant metas-
tases, particularly to distant organs such as the liver (Jackstadt and Sansom, 2016; Romano et al., 2019). For this reason, current models can be seen as excellent tools to study early-stage dis-
ease rather than malignant progression, with transplantation of tumor-derived organoids currently being the best alternative for analysis of metastatic spread (Romano et al., 2018; Tauriello et al., 2018). Transplantation models have highlighted a key role for LGR5+ stem cells in metastasis (de Sousa e Melo et al., 2017) and have suggested that TGF-β inhibitors may have efficacy in Apc-mutation-driven mCRC (Tauriello et al., 2018).

Activated NOTCH1 signaling has been observed in CRC and other cancer types (Sancho et al., 2015; Noah and Shroyer, 2013; Irshad et al., 2017). This activation can occur via NOTCH1 ligands on the surface of tumor cells or by components of the TME such as endothelial or innate immune cells (Meurette and Mehlen, 2018). Tumor cell-autonomous signaling can also occur by NOTCH1 receptor copy-number gain, reported in 22% of CRCs, with negative prognostic value (Arcaroli et al., 2016). In addition NOTCH1 signaling can be activated via mutation of FBXW7, found in 11% of human CRCs (Cancer Genome Atlas Network, 2012; Babaei-Jadidi et al., 2011). Activation of NOTCH1 signaling can contribute to cancer cell stemness, inva-
sion, and metastasis (Lu et al., 2013; Sonoshita et al., 2011, 2015; Rodilla et al., 2009; Wieland et al., 2017). Moreover, recent combination of activated NOTCH1 signaling and Trp53 deletion in the intestine resulted in metastatic disease, albeit with long la-
tency and relatively low penetrance (10% liver metastases) (Chanrion et al., 2014), limiting preclinical relevance. Importantly, the molecular mechanism driving NOTCH1-dependent metastasis and the requirement for additional oncogenic driver muta-
tions remains unclear.

There is an urgent need for improved therapeutic options for patients with advanced mCRC. Currently, molecular subtyp-
ing is the most effective strategy to identify patients with the poorest prognosis. For this reason, subtype-specific preclinical models are vital for development of new therapeutic approaches.

RESULTS

Mutation Context-Dependent Ability of NOTCH1 to Drive Intestinal Cancer Metastasis

Given associations between NOTCH signaling and CRC we generated a NOTCH-score (Kwon et al., 2016), based on expres-
sion of pathway components, and applied this to The Cancer Genome Atlas (TCGA) human CRC dataset (Cancer Genome Atlas Network, 2012). We found that a high NOTCH-score is significantly associated with CMS4 and poor prognosis (Figures S1A and S1B). Interestingly, when further stratified, the NOTCH-
score remained prognostic when KRAS was mutated (Fig-

ure S1C), and segregated the poorest prognosis patients in CMS4 (Figure S1D). In addition, we found a high percentage of human CRC metastasis strongly positive for NOTCH1 intracel-

lular domain (N1ICD), indicative of activated NOTCH1 signaling in human CRC metastasis (Figure S1E).

In light of these observations, we sought to test the functional role of NOTCH1 signaling in CRC. This was achieved using the
inducible enterocyte-specific villinCreER to recombine either one copy of Apcfl/+ or activate KrasG12D/+ in combination with deletion of Trp53fl/fl, and overexpression of the transcriptionally active N1ICD (Figure 1A). Consistent with previous studies, villinCreER Trp53fl/+ Rosa26N1icd/+ (PN) mice (Chanrion et al., 2014) developed tumors at long latency (Figure 1B). Importantly, all induced villinCreER KrasG12D/+ Trp53fl/+ Rosa26N1icd/+ (KPN) mice that developed intestinal adenocarcinoma exhibited metastases to lymph nodes, lungs, liver, and/or diaphragm at clinical endpoint (Figure 1C). A total of 83% (24/29) of KPN mice had liver metastases, recapitulating human disease (Figures 1D–1F). In contrast, APC-deficient models such as villinCreER Apcfl/+ Trp53fl/fl Rosa26N1icd/+ (APN) or villinCreER Apcfl/+ Trp53fl/fl (AP) did not develop metastases. PN or villinCreER KrasG12D/+ Trp53fl/fl (KP) mice developed few metastases and very rarely to distant sites: 5% liver and ~20% lymph nodes, respectively (Figure 1C). Expression of two copies of the N1icd allele (Figure S2A) or one copy of mutant Trp53fl/R172H in KPN mice did not change survival and/or metastatic burden (Figures S2B and S2C). Furthermore, mutations had only a mild impact on intestinal homeostasis (Figures S2D–S2G). Given that KP and KPN mice exhibited similar latency (Figure 1B), but only KPN mice displayed significantly increased metastatic burden (Figure 1C), we concluded that epithelial NOTCH1 drives metastasis in a setting where Trp53 is mutated and RAS/MAPK signaling is activated.
KPN Tumors Are of Serrated Origin
Human serrated CRCs have been associated with KRAS mutations (IJspeert et al., 2015), and these morphological features are reported to be recapitulated in the tumors of Kras<sup>St12D</sup>-driven intestinal GEMMs (Bennecke et al., 2010). Histological analysis of KPN tumors confirmed a serrated morphology of primary tumors, while tumors driven by Apc deletion exhibited a tubular morphology (Figure 2A). Consistent with the metastatic spread of KPN tumors, primary tumors were highly invasive and poorly differentiated, exhibited a high collagen content, significant infiltration of cancer associated fibroblasts (CAFs) and hypoxia, all features typical of advanced disease (Figures 2B–2D). On average, KPN mice developed two tumors per intestine (Figures 2E, 2F, and S3A). We analyzed the expression of the DNA mismatch repair protein MLH1 in primary tumors of APN and KPN mice. Retained expression of MLH1 indicates that these tumors are microsatellite stable (MSS) (Figure S3B). Therefore, KPN tumors represent models of MSS serrated intestinal cancer in which NOTCH1 signaling drives metastasis without impacting tumor initiation.

Alteration of WNT Signaling in the Metastatic KPN Model
Human serrated polyps show reduced WNT pathway activity compared with tubular adenomas which harbor APC mutations (Figure 3A) (Borowsky et al., 2018; Bennecke et al., 2010). Comparison of WNT target gene expression between GEMM primary tumors and human serrated adenoma (Fessler et al., 2016) revealed that KPs and KPNs are closely related to serrated tumors (Figure 3B). APN tumors exhibited significant activation of canonical WNT signaling, indicated by nuclear accumulation of β-catenin, with lower activation observed in KPN tumors (Figure 3C). This was reflected by distinct patterns of WNT target gene expression in each primary tumor type (Figures 3B, 3D, and S3B). Moreover, liver metastases from KPN tumors did not have a marked accumulation of nuclear β-catenin, although elevated expression of some WNT targets, including CD44 and SOX9, was observed (Figures 3C and S3C). This recapitulates activation of WNT seen in human serrated tumors and indicates that hyper-activation of epithelial canonical WNT signaling is not essential for metastasis.

Given these moderate levels of WNT signaling and reported upregulation of WNT ligands or R-spondins in CRC (Seshagiri et al., 2012), we examined ligand deregulation or ligand dependence in our models. RNA-sequencing data exhibited profound expression of WNT ligands (Figure S3D). To test ligand functionality we treated KPN mice with LGK974, a clinically relevant PORCUPINE inhibitor, from 85 days after induction, blocking WNT ligand secretion (Liu et al., 2013) (Figures 3E and S3E). This treatment had no impact on survival or metastatic rate (Figures 3F and 3G; Table S1). To understand the mechanism of WNT ligand independence, we derived organoid cultures from KPN primary tumors. When these organoids were passaged or seeded as single cells, they grew independently of WNT agonist R-spondin1 and were refractory to LGK974 treatment (Figures 3H–3J). Similarly, KP organoids were refractory to LGK974 treatment indicating a NOTCH1-independent mechanism (Figure S3F). This suggests an epithelial cell-intrinsic mechanism of WNT ligand-independent growth, or independence from WNT signaling altogether. To identify drivers of WNT activation, we applied whole-genome sequencing to ten KPN primary tumor-derived organoid lines. This approach confirmed loss of Trp53 and MSS status (overall 1.59 mutations/Mb; coding mutation rate 1.31 mutations/Mb) (Figures S4A–S4C and Table S2). Strikingly, four out of ten (40%) organoid lines had homozygous mutations in Csnk1a1 (encoding casein kinase 1α), a component of the β-catenin destruction complex (Figure 3K). Intriguingly, Csnk1a1 deletion in intestinal epithelial cells has been shown to trigger tumorigenesis only in combination with loss of Trp53 (Elyada et al., 2011). Furthermore, two lines showed Ephb2 missense mutations (Figure 3K), which has been associated with CRC progression (Batlle et al., 2005; Clevers and Batlle, 2006). Importantly, while these mutations drive increased expression of selected WNT targets, they appear to be weaker activators of the pathway than Apc loss and critically mimic levels found in human serrated tumors.

Epithelial NOTCH1 Drives Subtypes of Human CRC with Poorest Prognosis
To better understand how our model, and more broadly, NOTCH1 signaling relates to human CRC, we generated transcriptome-wide expression profiles from tumor tissue (consisting of epithelium and stroma). Comparison of signatures generated from both the serrated (KPN) and tubular (APN) tumors (Table S3) with human data revealed a poorer prognosis for patients resembling the KPN signature (Figure 4A), in line with the poor prognosis associated with human serrated CRC (De Sousa et al., 2013). Interestingly, when we analyzed organoid expression profiles derived from APN or KPN tumors (Table S4) this survival segregation still holds (Figure 4B), demonstrating the predictive value of epithelial KPN signatures. Comparison of mouse intestinal tumors with CMSs revealed a NOTCH1-dependent positive correlation between the KPN transcriptome and CMS4, and a negative correlation with CMS2/3 (Figure 4C). Strikingly, tumor models driven by Apc loss correlate with CMS2/3 (Figure 4C), which confers better disease prognosis (Guiney et al., 2015). Moreover, cross-comparison with CRIS signatures revealed that KPN tumors strongly correlate with CRIS-B (Figure 4D), associated with poor prognosis (Isella et al., 2017). Gene set enrichment analysis (GSEA) indicates that KPN tumors are enriched for CMS4/CRIS-B-associated signatures such as vascular endothelial growth factor/vascular endothelial growth factor receptor, EMT, and TGF-β activation (Figures 4E and S4D; Table S5). These data demonstrate that the GEMMs described here exhibit transcriptional overlap with the subset of human CRCs with poorest prognosis and that epithelial NOTCH1 is a key driver of those subtypes.

Epithelial NOTCH1 Controls Neutrophil Recruitment to Drive Metastasis
The current paradigm suggests that stromal signatures are associated with poor prognosis, which is particularly pertinent to the “mesenchymal” CMS4 tumor signature. In light of this, we have compared our transcriptional profiles with data used to characterize human CMS4 CRC as highly enriched for myeloid, angiogenic, inflammatory, fibroblast, and immunosuppressive cell signatures (Becht et al., 2016a, 2016b). While many of these features were recapitulated in metastatic KPN tumors (Figure 5A), most were also present in the non-metastatic KP model (Figures
Figure 2. Morphological Analysis of Primary Tumors
(A) Representative H&E images of primary tumors. Scale bars, 100 μm. Arrows indicate serrated morphology.
(B) Representative images of indicated markers on primary tumors. Scale bars, 100 μm.
(C and D) Tumor stage (C) and differentiation at endpoint (D).
(E) Macroscopic primary tumors per mouse.
(F) Macroscopic primary tumor burden per mouse.
In (C–F): AP, n = 10; APN, n ≥ 11; KP, n = 14; KPN, n ≥ 22. Error bars in (E and F) represent mean ± SEM. See also Figure S3.
Figure 3. Role of WNT Signaling in Metastatic KPN Tumors

(A) Expression of WNT targets in human tubular or serrated adenoma.
(B) Heatmap of a human serrated signature versus mouse primary tumor signatures. Scale bars, 100 μm. Right bottom: quantification of nuclear β-catenin in primary tumors (n ≥ 10).
(C) Quantification of in situ hybridization (ISH) for positive cells on primary tumors (APN, n ≥ 5; KPN, n ≥ 7).

(legend continued on next page)
5A and S4E), implying that they may not be determinants of metastatic spread. Importantly, enrichment of a neutrophil signature was associated with metastatic KPN tumors, but not with non-metastatic KP tumors (Figures 5B and S4E). Similar to human serrated adenoma, we have detected neutrophil accumulation in primary tumors, metastases, and systemically in KPN mice (Figures 5C–5F). Given that metastasis in the KPN model was associated with neutrophil infiltration, we assessed expression of chemokines implicated in neutrophil attraction (Figures 5G–5I).

To test this, we treated KPN mice with AZD5069, a clinically relevant CXCR2 small-molecule (CXCR2sm) inhibitor (Nicholls et al., 2015), which has been shown to block neutrophil recruitment. Importantly, while CXCR2sm treatment, from day 85 and S5A) finding increased expression of Cxcl5 in the epithelium of KPN tumors but not of KP (Figures 5G and 5H and Table S6). Cxcl5 expression was correlated with that of its receptor Cxcr2, which is predominantly expressed on neutrophils (Figure S5B). When we examined the expression of neutrophil-associated genes such as ELANE, MPO, and CXCR2 in human CRC, we found significantly increased expression in human CMS4 (Figure S5C). In addition, the neutrophil infiltration-score was able to predict survival in treatment-naive metastases and is significantly associated with CMS4 and CRIS-B (Figures S5D and S5E). Furthermore, neutrophil infiltration of metastases, analyzed by MPO or CXCR2 expression predicts poor survival in an additional cohort (Figure S5F; Table S6). For these reasons, we hypothesized that neutrophils may be a critical driver of NOTCH1-dependent metastasis in CMS4/CRIS-B CRC.

To test this, we treated KPN mice with AZD5069, a clinically relevant CXCR2 small-molecule (CXCR2sm) inhibitor (Nicholls et al., 2015), which has been shown to block neutrophil recruitment. Importantly, while CXCR2sm treatment, from day 85...
Figure 5. Epithelial NOTCH1 Controls Neutrophil Recruitment to Drive Metastasis
(A) Heatmap showing standardized infiltration-scores (calculated with MCPcounter) in GEMM tumors; AP, n = 3; APN, n = 3; KP, n = 3; KPN, n = 9.
(B) Dot-plots showing standardized infiltration scores of neutrophils (calculated with MCPcounter); replicates as in (A).
(D) Immunohistochemistry showing neutrophil infiltration in KPN tumors.
(F) Quantification of neutrophil infiltration in KPN tumors.
(I) Quantification of metastases in vehicle and CXCR2 inhibitor-treated groups.
(legend continued on next page)
post induction, did not impact survival or primary tumor burden in KPN mice, it profoundly reduced metastasis (Figures 5I, 6A, and 6B; Table S1). Short term treatment of KPN tumor-bearing mice with CXCR2sm resulted in reduced neutrophil counts in both the peripheral blood and primary tumors (Figures 5J and 5K), and an increase in CD8+ T cell numbers, compared with vehicle-treated counterparts (Figures 5K and S6C–S6E). The enhanced CD8+ T cell number, thought to create an anti-metastatic microenvironment at a secondary site, was retained in livers at endpoint (Figure S6F).

Given that CXCR2 expression may not be restricted to neutrophils, we evaluated the impact of neutrophil depletion with a Ly6G-targeting antibody (1A8). Again, metastasis was suppressed when compared with isotype control (2A3), but survival was unaffected (Figures 5I, S6G, and S6H; Table S1). Circulating neutrophils were reduced at endpoint, indicating a sustained effect of the neutralizing antibody (Figure S6I). We detected an increase in CD4+ and CD8+ T cells in the primary tumors of KPN mice treated with 1A8 in the short term (Figures 5K, S6J, and S6K), and increased CD8+ T cells in livers at endpoint (Figure S6L). Together, this indicates that epithelial NOTCH1 triggers CXCR2-dependent Ly6G+ neutrophil accumulation within the pre-metastatic niche and generates an immunosuppressive environment. Therapeutic targeting of neutrophils results in increased infiltrating CD8+ T cells within the pre-metastatic niche and a reduction in metastasis.

Epithelial NOTCH1 Signature Predicts Poor Survival and Drives Epithelial TGF-β2 Expression

To determine how epithelial NOTCH1 controls metastasis, we examined the transcriptome of tumor-derived KPN versus KP organoids (Figure 6A; Table S7). Increased expression of canonical NOTCH1 target genes, such as Fjx1 and Dtx1, along with an enriched NOTCH-score was observed in KPN organoids, compared with KP counterparts (Figures 6A and S7A). Strikingly in human CRC, the KPN/KP-score predicts poor prognosis and is associated with CMS4, CRIS-B, and neutrophil infiltration (Figures 6B and S7B–S7E). Interestingly, we found significantly increased expression of the gene encoding Tgfb2 ligand in KPN organoids (Figure 6A). TGFβ2 expression predicts poor survival and is significantly correlated with CRIS-B, CMS4, NOTCH-score, and KPN/KP-score in human CRC datasets (Figures 6B, 6C, and S7–S7J). Furthermore, TGFβ2 expression and the KPN/KP-score are also associated with human serrated adenoma (Figure 6D). Together, these data demonstrate a strong association between epithelial NOTCH1-dependent transcriptional signatures and high TGFB2 expression in serrated tumors which exhibit poor outcome and underscores the human relevance of the KPN model.

To understand how NOTCH1 controls Tgfb2 expression we confirmed NOTCH1-dependent expression of Tgfb2 in KPN organoids (Figure 6E). Moreover, GSEA showed that KPN primary tumors and organoids are associated with TGF-β1 activation (Figures 4E and 6F). Promoter analysis of the genomic area around the Tgfb2 transcriptional start site for putative RBPJ binding sites revealed a number of canonical RBPJ-DNA binding motifs (Figure 6G; Table S8). Chromatin immunoprecipitation for RBPJ, the key mediator of NOTCH1-mediated transcription, showed binding to the promoter of Tgfb2 in KPN organoids; however, no binding was detected to an upstream region of the Tgfb2 promoter which lacks RBPJ binding sites or a control region (Chm1) (Figure 6H).

Neutrophil Inhibition Attenuates Metastasis by T Cell Activation

We next examined the contribution that the epithelial compartment of KPN tumors makes to the TME in metastatic colonization. Previous studies have reported that intra-splenic transplantation of organoids from mouse intestinal tumors, with combined Apc, KrasG12D, Trp53, and TGF-β signaling mutations, is the most efficient means of generating metastases (Tauriello et al., 2018; Sakai et al., 2019) (Figure 7A). We found no difference in the capacity to colonize the liver between organoids derived from KPN or KP mice (Figures 5I, 7A). Tgfb1 expression was comparable in both primary tumors and organoids derived from KPN or KP mice (Figures 6A, 6E, and 6I), with predominantly stromal expression in KPN tumors (Figure S7K).

(C) Blood neutrophil count at endpoint of indicated genotype (n ≥ 6).
(D) Representative Ly6G IHC. Scale bars, 100 μm.
(E) Neutrophil infiltration-score in human adenoma.
(F) Quantification of Ly6G+ and S100A9+ cells per field of view (FOV); AP, n = 6; APN, n ≥ 5; KP, n ≥ 4; KPN, n ≥ 5.
(G) Representative ISH of Cxcl5 expression. Scale bars, 100 μm.
(H) Quantification of Cxcl5+ and Cxcr2+ cells; AP, n = 8; APN, n = 6; KP, n = 6; KPN, n ≥ 6.
(I) Incidence of metastases at endpoint for KPN mice treated with: vehicle, n = 11; CXCR2sm, n = 10; 2A3, n = 10; 1A8, n = 9; analyzed by chi-square test, two-tailed.
(J) Blood neutrophil count after 1 week of indicated treatments: vehicle, n = 5; CXCR2sm, n = 7; 2A3, n = 5; 1A8, n = 5; analyzed by Mann-Whitney U test, one-tailed.
(K) Quantification of IHC on primary tumors of KPN mice after 1 week of indicated treatments: vehicle, n ≥ 4; CXCR2sm, n = 7; 2A3, n = 5; 1A8, n = 5. Error bars in (B), (C), (E), (F), (H), (J), and (K) represent mean ± SEM. Data in (B), (C), (E), (F), (H), and (K) analyzed by Mann-Whitney U test, two-tailed. See also Figures S5 and S6 and Tables S1 and S6.
Figure 6. Epithelial NOTCH1 Drives Poor Prognosis Signatures and TGF-β2 Expression

(A) Volcano-plot of organoid KPN (n = 3) versus KP (n = 3) mRNA expression.
(B) RFS of CRC patients (TCGA), stratified using the KPN/KP-score as in (A) or TGFβ2 expression. The blue line shows expression ≤ median score (low), the red line shows expression > median score (high).
(C) Correlation of the KPN/KP-score and TGFβ2 expression in human serrated adenoma (top) or in TCGA data (bottom), p values were calculated by Pearson correlation.
(D) KPN/KP-score or expression of TGFβ2 in human adenoma.

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significant increase of circulating neutrophils (Figure S7M). Intriguingly, Alk5i treatment had no effect on circulating neutrophils but significantly reduced neutrophils infiltrating metastases (Figures 7H and 7I). In addition, we detected an increase in activated CD69+/CD44+, CD69+/CD8+, and IFNγ−CD4+ type 1 T helper (Th1) cells upon Alk5i treatment (Figure 7J).

Importantly, we detected increased CD8+ GzB+ cytotoxic T lymphocytes in metastases treated with Alk5i or CXC2R2sm (Figures 7J, S7N, and S7O). This suggests that the effect of CXC2R2sm and Alk5i on metastasis is mediated by alleviation of a neutrophil-dependent immunosuppressive microenvironment. This is supported by the finding that CXC2R2sm or Alk5i have no effects on metastasis when KPN organoids were transplanted into immune-deficient nude mice which lack T cells (Figure S7P). Together, these data show that the epithelial programs driven by NOTCH1 in KPN tumor cells rewire the TME and generate an immunosuppressive, pro-metastatic environment.

Inhibition of Neutrophil TGF-β Signaling Attenuates Metastasis

Given the NOTCH1-dependent expression of Tgfβ2 in the KPN model and the profound effect on metastasis in the transplantation model, we examined the importance of TGF-β signaling to NOTCH1-dependent metastasis. We confirmed TGF-β activity in KPN tumors via nuclear localization of phosphorylated SMAD3 (pSMAD3) and TGF-β signaling targets Smad7, CALD1, and IGFBP7 (Figure 8A). We then applied two independent, clinically relevant therapeutic approaches, either targeting of TGFBR1/ALK5 with Alk5i, or with a ligand-trapping antibody targeting TGF-β1/2/3 (1D11) (Figure 8B). In an early-intervention setting, in which mice are treated from 85 days after induction, inhibition of ALK5 resulted in rapid development of highly invasive (T3) colon tumors, although with markedly reduced metastasis (Figures 8B and 8C; Table S1). Interestingly, targeting the ligands with 1D11 did not result in accelerated tumorigenesis, but significantly reduced metastasis (Figures 8B and 8C; Table S1). In a late intervention approach, when mice were treated with Alk5i from 130 days after induction, developing tumors with a similar latency and comparable tumor burden as vehicle-treated mice (Figures 8B and S8A), but with significantly reduced metastatic penetrance at endpoint (Figure 8C; Table S1). This was associated with a significant reduction in the number of neutrophils in the liver (Figure 8D), with peripheral blood neutrophil counts being unaffected (Figure S8B). The reduction in liver neutrophils was accompanied by accumulation of CD3+, CD4+, and CD8+ T cells (Figures 8D, S8C, and S8D). Interestingly, no change in primary tumor fibrosis was detected when KPN mice were treated with Alk5i or 1D11 (Figure S8D). Taken together, these data support a strong role for TGF-β signaling in generating an immunosuppressive pro-metastatic microenvironment in the liver by recruiting neutrophils.

To address the specific function of TGF-β signaling in the neutrophil population of tumor-bearing mice, we transplanted KPN organoids into the colonic submucosa of syngeneic C57BL/6 mice lacking Alk5 expression specifically in Ly6G+ neutrophils (Ly6G-Cre Alk5fl/fl) (Figure 8E). Engrafted KPN organoids formed primary tumors that were morphologically indistinguishable from those of the autochthonous GEMM (Figures 8F, S8E, and S8F), with epithelial cells from primary tumors and liver metastases also found to express high levels of Tgfβ2 (Figure 8G). As predicted by inhibitor experiments, deletion of Alk5 in the neutrophil population had no beneficial impact on survival (Figure S8G), but led to a striking reduction in metastasis (Figure 8H). This was concomitant with reduced tumor-infiltrating neutrophils, although peripheral blood neutrophil counts were maintained (Figure 8I). Reduced neutrophil infiltration was not associated with an alteration in neutrophil maturity, as expression of CD101 (a marker of mature murine neutrophils) was unaffected by ALK5 deficiency (Figure 8J). Importantly, TGF-β signaling inhibition blocks metastasis by reducing neutrophil attraction, rather than by polarization to an anti-tumor phenotype.

DISCUSSION

The genetic progression of CRC has been investigated for many years, yet no robust drivers of metastasis have been identified in GEMMs. Importantly, we demonstrate that alteration of epithelial tumor cell-intrinsic signaling can rewire the TME and, in turn, promote metastasis. Notably, this occurs spontaneously only in concert with specific mutations that drive serrated tumors, and not during the progression of WNT-driven tubular adenoma. These observations are consistent with reported differential responses of tubular and serrated adenomas to TGF-β signaling (Fessler et al., 2016).

Whole-genome sequencing of tumors from KPN mice revealed relatively low levels of mutation, although recurrent biallelic mutations in Csnk1a1 were observed. This would suggest a further single sporadic mutation, for example in Csnk1a1, could drive rapid progression to adenocarcinoma and metastasis in the KPN model, possibly by generating an inflammatory TME (Pribluda et al., 2013). One could hypothesize that this recapitulates the “Big Bang” model of human CRC in which the key driver mutations occur early, while later, large tumors exhibit neutral evolution (Sottoriva et al., 2015).

The literature regarding NOTCH and WNT interaction in WNT-driven models reveals different roles of NOTCH1 signaling. Epithelial N1icd expression, in combination with Apc1263 allelic mutation, can drive tumor initiation (Fre et al., 2009), or inhibit
WNT signaling in the Apc<sup>Min/+</sup> model at the transcriptional level (Kim et al., 2012). Our results demonstrate that, in the context of Apc and Trp53 loss, N1icd has no significant impact on survival or tumor stage. It is interesting to note, however, that we observed reduced levels of selected WNT target genes in KPN tumors and that a specific WNT pathway mutation was selected in emerging tumors. Importantly, human serrated KRAS<sup>-mutant</sup> tumors predominantly exhibit low levels of nuclear β-catenin (Bennecke et al., 2010), mirrored in KPN tumors. This demonstrates that the model described here shares key cellular and molecular features with human serrated disease. We speculate that WNT ligand inhibition might be a therapeutic option for this type of CRC as reported for CRC with RNF43 mutations or R-spondin fusions (Storm et al., 2016; Yan et al., 2017; Han

**Figure 7. TGF-β or CXCR2 Inhibition Attenuates KPN Metastasis via T Cell Activation**

(A) Cartoon illustrating organoid isograft transplantation in the spleen.
(B) Quantification of macroscopic liver metastases 4 weeks post-transplantation; KPN, n = 4; AKPT, n = 5.
(C) Quantification of neutrophils in liver metastases by flow cytometry as in (B).
(D) Representative contour plots of the analysis performed in (C).
(E) Representative images for ISH analysis of Tgfb2 expression or IHC for Ly6G on liver metastases (n ≥ 3). Scale bars, 100 μm.
(F) Schematic representation of the treatment regimen after organoid transplantation.
(G) Number and burden of macroscopic liver metastases 4 weeks post-KPN organoid transplantation; vehicle, n = 5; Alk5i, n = 5.
(H and I) Quantification of flow cytometry analysis for neutrophils in blood (H) or liver metastases 4 weeks post-KPN organoid transplantation (I); vehicle, n = 5; Alk5i, n ≥ 4.

Error bars in (B), (C), (G), (H), (I), and (J) represent mean ± SEM. Data in (C), (G), (I), and (J) analyzed by Mann-Whitney U test, two-tailed. See also Figure S7.
This said, we found no response of these tumors to PORCUPINE inhibition. From our work, it is interesting to propose that the timing of WNT pathway activation is key. When Apc is lost early, adenoma/polyps are formed that require multiple further mutations to progress to adenocarcinoma (Sakai et al., 2018). In a serrated model, WNT pathway mutations occur much later (which happens in the KPN model) and drive rapid progression to carcinoma and metastasis.

Critically, our model suggests therapeutic targets in mCRC. NOTCH inhibitors are currently in non-stratified clinical trials for CRC and have shown some minor benefits (Andersson and Lendahl, 2014; Meurette and Mehlen, 2018); our data suggest benefits for NOTCH signaling inhibition in CMS4/CRIS-B patients. Combinatorial inhibition of MEK/ERK and γ-secretase increases efficacy in melanoma, papillary thyroid cancer, and CRC (Krepler et al., 2016; Yamashita et al., 2013; Schmidt et al., 2018). Identification of tumors with activated NOTCH1 signaling may be aided with the KPN/KP-score described here, or through use of TGF-β2 as surrogate. To generate the NOTCH1 signature we activated N1icd in intestinal enterocytes, mimicking the activation of NOTCH1 signaling in CRC by NOTCH1 receptor copy gain (Arcaroli et al., 2016). Activation of NOTCH1 in the KPN model had no impact upon normal homeostasis, possibly because of targeted activation of NOTCH1 in adult tissue rather than during embryonic development (Fre et al., 2005). Interestingly, lateral inhibition, which occurs in the normal small intestinal crypt to maintain the stem cell niche, could influence the dynamics of non-cell-autonomous NOTCH1 activation and may occur in malignant progression. This kind of non-cell-autonomous activation should be considered in the future.

Our work also elucidates a novel non-cell-autonomous role for NOTCH1 in CRC through control of chemokine expression (TGF-β2 and CXCL5). Interestingly, TGF-β2 shows a 100- to 500-fold higher affinity to betaglycan/TGFBRIII than TGF-β1/3 (Cheifetz et al., 1990). Previously, NOTCH1 signaling has been shown to impact the cellular secretome of multiple cancers (Hoare et al., 2016; Wieland et al., 2017; Shen et al., 2017). Interestingly, this cancer-associated secretome includes many inflammatory chemokines, similar to the KPN tumors, such as granulocyte-colony stimulating factor and interleukin-6. The potential release of numerous chemokines may help to explain the dramatic effects on neutrophils both in the blood and at metastatic sites in our model. Non-metastatic KP tumors develop a similarly fibroblast-rich stroma as KPN tumors, but lack TGF-β2 expression and neutrophil infiltration. Therefore, the TME generated by CAFs appears insufficient on its own to prime for metastasis but relies on the infiltration of neutrophils. This suggests that our GEMMs may differ from some of the recently described models in which CAFs were important in defining TGF-β sensitivity (Calon et al., 2012; Tauniello et al., 2018). Interestingly, these models were initiated by Apc loss (alongside Kras, Tp53, and Smad4/Tgbr1I1) and predominantly driven by transplantation or induced colitis. It would be interesting to examine the chemokine profiles of these tumors as they may well be differentially dependent on contributions from components of the TME, as indicated by the difference in TGF-β2 expression.

Though previously thought to be terminally differentiated cells, neutrophils exhibit phenotypic plasticity and adopt distinct mature phenotypes (Leach et al., 2019). Accordingly, neutrophils have recently been shown to express various markers of maturity (Evrard et al., 2018). In particular, TGF-β signaling is thought to be a key regulator of the pro-tumorigenic state of neutrophils, and TGF-β inhibition has been shown to drive increased tumor infiltration of neutrophils (Frieldender et al., 2009). This is in contrast to our findings, where pharmacological inhibition of TGF-β signaling and genetic deletion of TGF-β signaling activity in neutrophils reduces neutrophil numbers in tumors but has no effect on neutrophil maturation status.

Future studies should characterize the phenotypic traits of neutrophils in the metastatic niche, when compared with the primary tumor and peripheral blood. Our findings suggest that CXCR2/ALK5-expressing neutrophils are important in CMS4/CRIS-B CRC disease progression and in the genesis of CRC metastases. The unresponsiveness of primary tumors to CXCR2sm treatment pre-clinically in this study and other studies (Steel et al., 2016) might be explained by active immune checkpoints in the primary site, which are lacking in the metastatic site. It should be noted that our mouse model shows high systemic levels of neutrophils and that clinically high neutrophil-to-lymphocyte ratios (NLR) are often associated with the poorest prognosis (Roxburgh et al., 2010). It would be of interest to determine whether these NLRs could be used to stratify patients for CXCR2/TGF-β/NOTCH-targeted therapies. In particular, the sensitivity of metastases to neutrophil inhibition holds promise as a potential treatment option for stage II/III CRC patients with undergoing primary tumor resection before treatment with CXCR2 or ALK5 inhibitors. Critically, we highlight a novel targeted therapeutic approach which may compromise the seeding of metastases in a setting in which major primary...
and metastatic tumor burden is reduced through surgical resection.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

R.J., S.R.v.H., J.P.M., and O.J.S. designed the research. R.J., J.O.L., S.R.v.H., X.C.-L., C.Nixon, J.O.L., R.A.R., V.M.W., W.C., C.Nourse, M.R., and A.H. performed the experiments and analyzed the data. C.W.S., T.J.K., C.S.R., P.G.H., A.D.C., D.J.A., P.B., A.V.B., M.G., J.R., O.H.Y., and S.T.B. contributed new reagents and/or analytical tools. R.J. and O.J.S. wrote the paper, all authors read the manuscript and provided critical comments.

DECLARATION OF INTERESTS

Simon T. Barry is an employee and shareholder of AstraZeneca.

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REFERENCES

Andera, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol. 11, R106.
Andersson, E.R., and Lendahl, U. (2014). Therapeutic modulation of Notch signalling—are we there yet? Nat. Rev. Drug Discov. 13, 357–378.
Anderton, M.J., Mellor, H.R., Bell, A., Sadler, C., Pass, M., Powell, S., Steele, S.J., Roberts, R.R., and Heier, A. (2011). Induction of heart valve lesions by small-molecule ALK5 inhibitors. Toxicol. Pathol. 39, 916–924.
Arcaroli, J.J., Tai, W.M., McWilliams, R., Bagby, S., Blatchford, P.J., Varella-Garcia, M., Purkey, A., Quackenbush, K.S., Song, E.K., Pitts, T.M., et al. (2016). A NOTCH1 gene copy number gain is a prognostic indicator of worse survival and a predictive indicator to a Notch1 targeting antibody in colorectal cancer. Int. J. Cancer 173, 195–205.
Babaei-Jadidi, R., Li, N., Saadeddin, A., Spencer-Dene, B., Jandke, A., Muhammad, B., Ibrahim, E.E., Muralleedharan, R., Abuzinadah, M., Davis, H., et al. (2011). FBXW7 influences murine intestinal homeostasis and cancer targeting Notch, Jun, and DEK for degradation. J. Exp. Med. 208, 295–312.
Battle, E., Bacani, J., Begthel, H., Jonkheer, S., Gregorieff, A., van de Born, M., Malats, N., Sancho, E., Boon, E., Pawson, T., et al. (2005). EphB receptor activity suppresses colorectal cancer progression. Nature 435, 1126–1130.
Becht, E., De Reynies, A., Giraldo, N.A., Plati, C., Buttard, B., Lacroix, L., Selves, J., Sauves-Fridman, C., Laurent-Puig, P., and Fridman, W.H. (2016b). Immune and stromal classification of colorectal cancer is associated with molecular subtypes and relevant for precision immunotherapy. Clin. Cancer Res. 22, 4057–4066.
Becht, E., Giraldo, N.A., Lacroix, L., Buttard, B., Ehtarouci, N., Pettitez, F., Selves, J., laurent-Puig, C., Fridman, W.H., and de Reynies, A. (2016b). Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. Genome Biol. 17, 218.
Bennecke, M., Kriegi, L., Bajbouj, M., Retzlaff, K., Robine, S., Jung, A., Arkan, M.C., Kirchner, T., and Greten, F.R. (2010). Ink4a/Arf and oncogene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis. Cancer Cell 18, 135–146.
Boeva, V., Popova, T., Bleakley, K., Chiiche, P., Cappo, J., Schielemacher, G., Janoueix-Lerosey, I., Delattre, O., and Barillot, E. (2012). Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. Bioinformatics 28, 423–425.
Borowsky, J., Dumenil, T., Bettington, M., Pearson, S.A., Bond, C., Fennell, L., Liu, C., McKeone, D., Rosty, C., Brown, I., et al. (2018). The role of APC in WNT pathway activation in serrated neoplasia. Mod. Pathol. 31, 495–504.
Calon, A., Espinet, E., Palomo-Ponce, S., Tauriello, D.V., Iglesias, M., Cespedes, M.V., Sevillano, M., Nadal, C., Jung, P., Zhang, X.H., et al.
Chanrion, M., Kuperstein, I., Barriere, C., el Marjou, F., Cohen, D., Vignjevic, D., Cancer Genome Atlas Network. (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337.

Carlson, M. 2017. org.Hs.eg.db: Genome wide annotation for Human. R package version 3.4.0. 10.18129/B9.bioc.org.Hs.eg.db

Chanrion, M., Kuperstein, I., Barriere, C., el Marjou, F., Cohen, D., Vignjevic, D., Stimmer, L., Paul-Gilloteaux, P., Bieche, I., Tavares Sdos, R., et al. (2014). Concomitant Notch activation and p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. Nat. Commun. 5, 5005.

Chiefetz, S., Hernandez, H., Laiho, M., Ten Dijke, P., Iwata, K.K., and Massague, J. (1990). Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. J. Biol. Chem. 265, 20533–20538.

Church, D.M., Schneider, V.A., Graves, T., Auger, K., Cunningham, F., Bouk, N., Chen, H.C., Agarwala, R., McLaren, W.M., Ritchie, G.R., et al. (2011). Modernizing reference genome assemblies. PLoS Biol. 9, e1001091.

Clevers, H., and Batlle, E. (2006). EphB/EphrinB receptors and Wnt signaling in colorectal cancer. Nature Rev. Gastroenterol. Hepatol. 12, 401–409.

Irshad, S., Bansal, M., Guarnieri, P., Davis, H., Al Haj Zen, A., Baran, B., Pinna, C.M.A., Rahman, H., Biswas, S., Bardella, C., et al. (2017). Bone morphogenetic protein and Notch signaling crosstalk in poor-prognosis, mesenchymal-subtype colorectal cancer. J. Pathol. 242, 178–192.

Jackson, E.L., Willis, N., Mercer, K., Bronson, R.T., Crowley, M., Montoya, R., Jacks, T., and Tuveson, D.A. (2001). Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev. 15, 3243–3248.

Jackson, R.T., and Sansom, O.J. (2016). Mouse models of intestinal cancer. J. Pathol. 238, 141–151.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. CA Cancer J. Clin. 61, 69–90.

Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. 29, 5005.

Keane, T.M., Goodstadt, L., Danecek, P., White, M.A., Wong, K., Yalcin, B., Heger, A., Agam, A., Slater, G., Goodson, M., et al. (2011). Mouse genomic variation and its effect on phenotypes and gene regulation. Nature 477, 289–294.

Kim, H.A., Koo, B.K., Cho, J.H., Kim, Y.Y., Seong, J., Chang, H.J., Oh, Y.M., Stange, D.E., Park, J.G., Hwang, D., and Kong, Y.Y. (2012). Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. J. Clin. Invest 122, 3248–3259.
Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Krepler, C., Xiao, M., Samanta, M., Vuttur, A., Chen, H.Y., Bradford, P., Reyes-Unrue, P.I., Halloran, M., Chen, T., He, X., et al. (2016). Targeting Notch enhances the efficacy of ERK inhibitors in BRAF-V600E melanoma. Oncotarget 7, 71211–71222.

Kwon, O.J., Zhang, L., Wang, J., Su, Q., Feng, Q., Zhang, X.H., Mani, S.A., Pautler, R., Creighton, C.J., Ittmann, M.M., and Xin, L. (2016). Notch promotes abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I insertions, deletions and gene fusions. Genome Biol. 17, R36.

Krepler, C., Xiao, M., Samanta, M., Vuttur, A., Chen, H.Y., Bradford, P., Reyes-Unrue, P.I., Halloran, M., Chen, T., He, X., et al. (2016). Targeting Notch enhances the efficacy of ERK inhibitors in BRAF-V600E melanoma. Oncotarget 7, 71211–71222.

Kwon, O.J., Zhang, L., Wang, J., Su, Q., Feng, Q., Zhang, X.H., Mani, S.A., Pautler, R., Creighton, C.J., Ittmann, M.M., and Xin, L. (2016). Notch promotes tumor metastasis in a prostate-specific Pten-null mouse model. J. Clin. Invest 126, 2626–2641.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Larsson, J., Goumans, M.J., Sjostrand, L.J., Van Rooijen, M.A., Ward, D., Leveen, P., Xu, X., Ten Dijke, P., Mummery, C.L., and Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J. 20, 1683–1693.

Leach, J., Morton, J.P., and Sansom, O.J. (2019). Neutrophils: homing in on the myeloid mechanisms of metastasis. Mol. Immunol. 106, 69–76.

Leek, J.T., Johnson, W.E., Parker, H.S., Pimentel, H., Kelley, R., and Salzberg, S.L. (2015). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 31, 2048–2056.

Liu, J., Pan, S., Hsieh, M.H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A.G., Li, A.G., Cheng, D., et al. (2013). Targeting Wnt-driven cancer through the Wnt/beta-catenin pathway in cancer models of Li-Fraumeni syndrome. Cell 152, 71211–71222.

Liu, J., Pan, S., Hsieh, M.H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A.G., Li, A.G., Cheng, D., et al. (2013). Targeting Wnt-driven cancer through the Wnt/beta-catenin pathway in cancer models of Li-Fraumeni syndrome. Cell 152, 71211–71222.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Lu, J., Ye, X., Fan, F., Xia, L., Bhattacharya, R., Bellister, S., Tozzi, F., Sceusi, E., Zhou, Y., Tachibana, I., et al. (2013). Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. Cancer Cell 23, 171–185.

el Marjou, F., Janssen, K.P., Zhang, B.H., Vellenga, E., Zhou, Y., Tachibana, I., et al. (2013). Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. Cancer Cell 23, 171–185.

McCorry, A.M., Loughrey, M.B., Longley, D.B., Lawler, M., and Dunne, P.D. (2018). Epithelial-to-mesenchymal transition signature assessment in colorectal cancer quantifies tumour stromal content rather than true tissue invasion. J. Pathol. 246, 422–436.

Meurette, O., and Mehen, R. (2018). Notch signaling in the tumor microenvironment. Cancer Cell 34, 536–548.

Murtagh, L.C., Stanger, B.Z., Kwan, K.M., and Melton, D.A. (2003). Notch signaling controls multiple steps of pancreatic differentiation. Proc. Natl. Acad. Sci. U S A 100, 14920–14925.

Nicholls, D.J., Wiley, K., Dainty, I., Macintosh, F., Phillips, C., Gaw, A., and Nicholls, D.J., Wiley, K., Dainty, I., Macintosh, F., Phillips, C., Gaw, A., and Mardh, C.K. (2015). Pharmacological characterization of AZD5068, a slowly reversible CXC chemokine receptor 2 antagonist. J. Pharmacol. Exp. Ther. 353, 340–350.

Noah, T.K., and Shroyer, N.F. (2013). Notch in the intestine: regulation of the myeloid cells of the intestinal immune portal: an innovative alternative to large, centralized data repositories. Nucleic Acids Res. 43, W589–W598.

Sottoriva, A., Kang, H., Ma, Z., Arora, T., O’Byrne, K.J., et al. (2015). A Big Bang model of human colorectal tumor growth. Nat. Genet. 47, 209–216.

de Sousa e Melo, F., Kurtova, A.V., Harnoss, J.M., Klajin, N., Hoeck, J.D., Hung, J., Anderson, J.E., Storm, E.E., Modrusan, Z., Koeppen, H., et al. (2015). Stem cell and progenitor fate in the mammalian intestine: notch and lateral inhibition in homeostasis and disease. EMBO Rep. 16, 571–581.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., Van Es, J.H., Abe, K., Kuji, A., Peters, P.J., and Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262–265.

Schmidt, E.M., Lamprecht, S., Blaj, C., Schauf, C., Krebs, S., Blum, H., Hermeking, H., Jung, A., Kirchner, T., and Horst, D. (2018). Targeting tumor cell plasticity by combined inhibition of NOTCH and MAPK signaling in colon cancer. J. Exp. Med. 215, 1693–1708.

Sancho, R., Cremona, C.A., and Behrens, A. (2015). Stem cell and progenitor fate in the mammalian intestine: notch and lateral inhibition in homeostasis and disease. EMBO Rep. 16, 571–581.

Sotomayor, E.M., Ishii, N., Aoki, M., Sasaki, Y., Kiyokawa, Y., et al. (2011). Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell 19, 125–137.

Sotomayor, E.M., Ishii, N., Aoki, M., Sasaki, Y., Kiyokawa, Y., et al. (2011). Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell 19, 125–137.

Sonoda, H., Takahashi, T., Shimizu, Y., and Whitehead, S., Thompson, P., Berman, H., Zuniga-Pflucker, J.C., et al. (2017). Notch shapes the innate immunophenotype in breast cancer. Cancer Discov. 7, 1320–1335.

Sottoriva, A., Kang, H., Ma, Z., Graham, T.A., Salomon, M.P., Zhao, J., Marjoram, P., Siegmund, K., Press, M.F., Shibata, D., and Curtis, C. (2015). A Big Bang model of human colorectal tumor growth. Nat. Genet. 47, 209–216.
(2017). A distinct role for Lgr5(+) stem cells in primary and metastatic colon cancer. Nature 543, 676–680.

De Sousa, E.M.F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P., de Jong, J.H., de Boer, O.J., van Leersum, R., Bijlsma, M.F., et al. (2013). Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat. Med. 19, 614–618.

Steele, C.W., Karim, S.A., Leach, J.D., Bailey, P., Upstill-Goddard, R., Rishi, L., Foth, M., Bryson, S., McDaid, K., Wilson, Z., et al. (2016). CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma. Cancer Cell 29, 832–845.

Storm, E.E., Durinck, S., de Sousa e Melo, F., Tremayne, J., Klijavin, N., Tan, C., Ye, X., Chiu, C., Pham, T., Hongo, J.A., et al. (2016). Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. Nature 529, 97–100.

Tauriello, D.V., Calon, A., Lonardo, E., and Battie, E. (2017). Determinants of metastatic competency in colorectal cancer. Mol. Oncol. 11, 97–119.

Tauriello, D.V.F., Palomo-Ponce, S., Stork, D., Berenguer-Llergo, A., Badia-Ramentol, J., Iglesias, M., Sevillano, M., Ibiza, S., Canellas, A., Hernando-Momblona, X., et al. (2018). TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis. Nature 554, 538–543.

Trobridge, P., Knoblaugh, S., Washington, M.K., Munoz, N.M., Tsuchiya, K.D., Rojas, A., Song, X., Ulrich, C.M., Sasazuki, T., Shirasawa, S., and Grady, W.M. (2009). TGF-beta receptor inactivation and mutant Kras induce intestinal neoplasms in mice via a beta-catenin-independent pathway. Gastroenterology 136, 1680–1688.e7.

Wellenstein, M.D., and de Visser, K.E. (2018). Cancer-cell-intrinsic mechanisms shaping the tumor immune landscape. Immunity 48, 399–416.

Wieland, E., Rodriguez-Vita, J., Liebler, S.S., Mogler, C., Moll, I., Herberich, S.E., Espinet, E., Herpel, E., Menuchin, A., Chang-Claude, J., et al. (2017). Endothelial Notch1 activity facilitates metastasis. Cancer Cell 37 (3), 355–367.

Yamashita, A.S., Geraldo, M.V., Fuziwara, C.S., Kulcsar, M.A., Friguglietti, C.U., da Costa, R.B., Baia, G.S., and Kimura, E.T. (2013). Notch pathway is activated by MAPK signaling and influences papillary thyroid cancer proliferation. Transl Oncol. 6, 197–205.

Yan, H.H.N., Lai, J.C.W., Ho, S.L., Leung, W.K., Law, W.L., Lee, J.F.Y., Chan, A.K.W., Tsui, W.Y., Chan, A.S.Y., Lee, B.C.H., et al. (2017). RNF43 germline and somatic mutation in serrated neoplasia pathway and its association with BRAF mutation. Gut 66, 1645–1656.

Ye, K., Schulz, M.H., Long, Q., Apweiler, R., and Ning, Z. (2009). Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics 25, 2865-2871.
# STAR Methods

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| TruStain FcX⁺ (anti-mouse CD16/32 Antibody) | Biolegend | 101320; RRID: AB_1574975 |
| CD45 Monoclonal Antibody (30-F11), Super Bright 600 | ThermoFisher | 63-0451-82; RRID: AB_2637149 |
| PE/Cy7 anti-mouse CD48 Antibody | Biolegend | 103424; RRID: AB_2075049 |
| Brilliant Violet 785⁺ anti-mouse/human CD11b Antibody | Biolegend | 101243; RRID: AB_2561373 |
| BUV395 Rat Anti-Mouse Ly-6G | BD Biosciences | 563978; RRID: AB_2716852 |
| CD101 Monoclonal Antibody (Moushi101), PE | ThermoFisher | 12-1011-82; RRID: AB_1210728 |
| Brilliant Violet 421⁺ anti-mouse CD3 Antibody | Biolegend | 100228; RRID: AB_2562553 |
| Alexa Fluor® 700 anti-mouse CD8a Antibody | Biolegend | 100730; RRID: AB_493703 |
| Alexa Fluor® 647 anti-human/mouse Granzyme B Antibody | Biolegend | 515406; RRID: AB_2566333 |
| IFN gamma Monoclonal Antibody (XMG1.2), PE-Cyanine7 | ThermoFisher | 25-7311-41; RRID: AB_1257211 |
| PE anti-mouse CD69 Antibody | Biolegend | 104508; RRID: AB_313111 |
| Anti-CD3 antibody [SP7] | Abcam | Ab16669; RRID: AB_443425 |
| CD4 Monoclonal Antibody (4SM95) | eBioscience | 14-9766-82; RRID: AB_2573008 |
| CD8a Monoclonal Antibody (4SM15) | eBioscience | 14-0808-82; RRID: AB_2572861 |
| Calgranulin B Antibody (M-19) | Santa Cruz | sc-8115; RRID: AB_2269986 |
| Purified Rat Anti-Mouse CD44 Clone IM7 | BD Biosciences | 550538; RRID: AB_393732 |
| Anti-Sox9 Antibody | Millipore | AB5535; RRID: AB_2239761 |
| Purified Mouse Anti-β-Catenin Clone 14 | BD Biosciences | 610154; RRID: AB_397555 |
| Anti-MLH1 antibody [EPR3894] | Abcam | ab29312; RRID: AB_2049968 |
| Anti-CALD1 antibody produced in rabbit | Sigma-Aldrich | HPA008066; RRID: AB_1078378 |
| Anti-IGFBP7 antibody produced in rabbit | Sigma-Aldrich | HPA002196; RRID: AB_1079107 |
| Anti-Smad3 (phospho S423 + S425) antibody | Abcam | Ab52903; RRID: AB_882596 |
| Monoclonal Anti-Actin, α-Smooth Muscle | Sigma-Aldrich | A2547; RRID: AB_476701 |
| 4.3.11.3 mouse MAb | Hypoxyprobe | HP1-100; RRID: AB_2801307 |
| InVivoMAB anti-mouse Ly6G | Bioxcell | BE0075-1; RRID: AB_1107721 |
| InVivoMAB Rat IgG2a isotype control; clone 2A3 | Bioxcell | BE0088; RRID: AB_1107769 |
| InVivoMAB TGF-β ligand-antibody; clone 1D11 | Bioxcell | BE0057; RRID: AB_1107757 |
| InVivoMAB TGF-β ligand-antibody isotype control; clone MOPC-21 | Bioxcell | BE0083; RRID: AB_1107784 |
| CXCR2 Polyclonal Antibody | ThermoFisher | PA1-20673; RRID: AB_2126489 |
| Myeloperoxidase (MPO) | Dako | A0398; RRID: AB_2335676 |
| Cleaved Notch1 (Val1744) (D3B8) Rabbit mAb | Cell Signaling Technology | 4147; RRID: AB_2153348 |
| RBPSUH (D10A4) XP® Rabbit mAb | Cell Signaling Technology | 5313; RRID: AB_2665555 |
| Normal Rabbit IgG | Cell Signaling Technology | 2729; RRID: AB_1031062 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| CXCR2 inhibitor; AZD5069 | AstraZeneca | N/A |
| Akt5 inhibitor; AZ1260111 | AstraZeneca | N/A |
| LGK974 | Active Biochem | A-1400 |
| Permeabilization Buffer (10X) | Invitrogen | 00-8333-56 |
| Arc⁺ Amine Reactive Compensation Bead Kit | ThermoFisher | A10346 |
| UltraComp eBeads⁺ Compensation Beads | ThermoFisher | 01-2222-41 |
| GentleMACS C Tubes | Miltenyi Biotec | 130-093-237 |
| Mouse Tumor Dissociation Kit | Miltenyi Biotec | 130-096-730 |
| LIVE/DEAD⁺ Fixable Near-IR Dead Cell Stain Kit | ThermoFisher | L10119 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for further information, reagents, and resources should be directed to and will be fulfilled by the Lead Contact, Owen J. Sansom: (o.sansom@beatson.gla.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Species used: Mus musculus

Tumor Models and Treatments
All animal experiments were performed in accordance with a UK Home Office licence (Project License 70/8646), adhered to ARRIVE guidelines and were subject to review by the animal welfare and ethical review board of the University of Glasgow. Both genders were induced with a single injection of 2 mg tamoxifen (Sigma-Aldrich, T5648) by intraperitoneal injection at an age of 6 to 12 weeks, all experiments were performed on a C57BL/6 background (N = 5 or more). Mice were sampled at clinical endpoint, which was defined...
as weight loss and/or hunching and/or cachexia. Mice were censored ≥ 550 days after tamoxifen administration or if sampled not due to intestinal tumor burden or associated metastasis. The alleles used can be found in the Key Resources Table.

Alk5 inhibitor (Alk5i) (Anderton et al., 2011) (AstraZeneca, AZ12601011) was administered at 50 mg/kg and CXCR2 small molecule (AstraZeneca, AZD5068) at 100 mg/kg, both in 0.5% Hydroxypropyl Methylcellulose (HPMC) and 0.1% Tween-80 twice daily by oral gavage. As vehicle control for Alk5i and CXCR2sm 0.5% HPMC and 0.1% Tween-80 was given with the same regime. Ly6G-antibody (clone 1A8, BioXcell, BE0075-1) or isotype control (clone 2A3, BioXcell, BE0083) were administered three times a week by intraperitoneal injection at 10 mg/kg. TGF-β ligand-antibody (clone 1D11, BioXcell, BE0057) or isotype control (clone MOPC-21, BioXcell, BE0083) were administered three times a week by intraperitoneal injection at 5 mg/kg. LGK974 (Active Biochem, A-1400) was administered at 5 mg/kg, in 0.5% Methylcellulose (MC) and 0.5% Tween-80 twice daily by oral gavage. As vehicle control 0.5% MC and 0.5% Tween-80 was given with the same regime. Treatments were started 85 or 130 days after initial tamoxifen injection; short term treatments of tumor bearing mice were started when tumors were palpable.

**Patient Material**

46 patients who underwent synchronous resection of colorectal primary tumor and liver metastases between April 2002 and June 2010 at Glasgow Royal Infirmary were included in the study, details can be found in Table S6. Patients were identified from a prospectively maintained database and represent a consecutive cohort of resected patients. Application to access patient tissue was approved by the NHS Greater Glasgow and Clyde biorepository and ethical approval granted in biorepository application #357 and informed consent was obtained from all subjects. Patients were followed up at one month, six monthly until two years, and thereafter annually until five years at which point they were discharged. Recurrence data, morbidity, and mortality was prospectively collected. Information on date and cause of death was determined via access to the NHS Greater Glasgow and Clyde clinical portal. Death records were complete until 1st November 2017, which served as the censor date.

Human liver metastases were anonymised, five micron-thick, formalin fixed and paraffin embedded sections of liver containing metastatic colorectal carcinoma from partial hepatectomy specimens were stained for N11CD. The use of the human material was approved by the Lothian NRS Human Annotated Bioresource and informed consent was obtained from all subjects (ethical review number 15/ES/0094).

**METHOD DETAILS**

**Scoring of Tumor Stage and Differentiation**

T staging of tumors was performed by a boarded pathologist according to the following parameters included in the classical TNM classification; T0, no evidence of primary tumor. Tis, Carcinoma in situ: intraepithelial or invasion of the lamina propria (i.e. no extension through the muscularis mucosae and therefore the submucosa). T1, Tumor invades submucosa. T2, Tumor invades muscularis propria. T3, Tumor invades into the subserosa. T4, Tumor invades/perforates the visceral peritoneum and into other adjacent organs/structures.

Tumor differentiation scoring was performed by a boarded pathologist according to the following parameters; well differentiated tumors exhibit clear glandular differentiation in >95% of the tumor. Moderately differentiated tumors exhibit glandular differentiation in 50-95% of the tumor. Poorly differentiated carcinomas exhibit glandular differentiation in 5-50% of the tumor.

**Blood Count Analysis**

Blood was collected in EDTA columns after cardiac puncture. Blood samples were analyzed with IDEXX ProCyte Dx.

**Organoid Culture**

Advanced DMEM/F12 was supplemented with penicillin/streptomycin (100 U/ml / 100 µg/ml) (15140122), 2 mM L-Glutamine (25030081), 10 mM HEPES (15630080), N2-supplement (17502001) and B27-supplement (17504044) (all ordered from Gibco, Life Technologies or ThermoFisher-Scientific) and from here on is referred to as ADF. Complete ADF was prepared by supplementing ADF with 50 ng/ml Recombinant Human EGF (Peprotech, AF-100-15), 100 ng/ml Recombinant Murine Noggin (Peprotech, 25030081), 10 mM HEPES (15630080), N2-supplement (17502001) and B27-supplement (17504044) (all ordered from Gibco, Life Technologies or ThermoFisher-Scientific) and from here on is referred to as ADF. Complete ADF was prepared by supplementing ADF with 50 ng/ml Recombinant Human EGF (Peprotech, AF-100-15), 100 ng/ml Recombinant Murine Noggin (Peprotech, 250-38) and 500 ng/ml Recombinant Mouse R-spondin-1 (R&D systems, 3474-RS). Intestinal epithelium extraction (Faller et al., 2015) and culture conditions were previously described (Sato et al., 2009). These culture conditions were used unless stated differently in figure legends.

Tumors were cut into small fragments and washed five times in PBS. Tumor fragments were incubated in 5 ml 10x Trypsin (5mg/ml, Gibco), 1x DNase buffer and 200U recombinant DNase I (Roche, 04716278001) at 37°C for 30 minutes. To further dissociate tumor fragments, 5 ml ADF was added and tumor fragments were shaken vigorously. This step was repeated five times. After aspirating the supernatant and re-suspending the pellet in 10 ml ADF, the suspension was passed through a 70 µm cell strainer. The cell pellet was re-suspended in Matrigel (BD Bioscience, 356231) according to pellet volume and seeded. Organoids/spheroids were cultured in complete ADF at 37°C, 5% CO2, 21% O2.

**Single Cell Seeding**

Organoids were harvested and dissociated by fiercely pipetting. Organoids were washed twice with PBS before being dissociated into single cells by incubating in 2 ml 10x Trypsin (5mg/ml), 1x DNase buffer and 200U recombinant DNase I (Roche, 04716728001) at
Cells were harvested and washed with PBS and trypsinized with 0.25% Trypsin in PBS-EDTA for 7 minutes at 37°C (AKPT; small intestinal derived; C57BL/6, N=7) organoids were cultured in conditions as described above without R-spondin. Tumor cells were harvested and washed with PBS and trypsinized with 0.25% Trypsin in PBS-EDTA for 7 minutes at 37°C. After trypsinization, cells were washed and passed through a 40 μm cell strainer and counted using a haemocytometer.

C57BL/6 or CD-1/Nude mice (6-12 weeks old males; Charles River, UK) were anesthetized with isoflurane, and a laparotomy was performed to gain access to the spleen. 5x10⁵ single cells in 50 μL PBS were injected into the spleen after which the incision was sutured. The mice were sampled four weeks post transplantation. Organoid donor and recipient mice were sex matched.

Needle-Guided Intracolonic Organoid Transplantation
Colonic sub-mucosal injections of organoids were performed as previously described (Roper et al., 2017), using a Karl Storz TELEPACK VET X LED endoscopic video unit. KPN liver metastases derived (C57BL/6, N=10) organoids, cultured in conditions as described above without R-spondin, were harvested and dissociated by fiercely pipetting. Organoids were washed twice with PBS before being injected. Approximately 500 organoids in 70 μL PBS were injected in a single injection. At clinical end point tumors and metastasis were quantified.

Sample Processing and Staining for Flow Cytometry
Tumor samples were dissected and digested using the Mouse Tumor Dissociation Kit (Miltenyi Biotec, 130-096-730) and the GentleMACS Octo Dissociator with Heaters (Miltenyi Biotec, 130-096-427), using the 37°C m TDK 1 programme. The cells were passed through a 70 μm cell strainer and then counted. Two million cells were stained with LIVE/DEAD fixable near-IR stain kit (ThermoFisher, L10119) at 1:1000 dilution in 100 μL PBS in the dark for 20 minutes at 4°C, then washed with PBS 1% BSA. TrueStain FcX anti-mouse CD16/32 (Biolegend, 101243), Ly6G (BD Biosciences, 563978), and CD101 (ThermoFisher, 12-1011-82) at 1:1000 dilution in 100 μL PBS were injected in a single injection. At clinical end point tumors and metastasis were quantified.

DNA degradation step using the Qiagen RNase-Free DNase kit (Qiagen, 79254). Cell pellets or tissue were lysed using the Precellys Evolution machine (Bertin Instruments). RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, 74104) according to the manufacturer’s protocol including the optional RNA Isolation

RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, 74104) according to the manufacturer’s protocol including the optional DNA degradation step using the Qiagen RNAse-Free DNase kit (Qiagen, 79254). Cell pellets or tissue were lysed using the Precellys lysing kit (Bertin Instruments, KT03961-1-003-2) in a Precellys Evolution machine (Bertin Instruments).

Organoids of the respective genotype, at comparable passage ~ 5 were sampled 72 hours post seeding. Organoid pellets were snap-frozen and RNA was isolated as described above.

RNA of whole tumor samples was isolated at endpoint from genotypes as indicated in figure legends and conserved in RNAlater (Sigma, R0901) at -80°C until further use for RNA isolation as described above. For sequencing tumor fragments were excised from the tumor centre to minimize effects of intra-tumor heterogeneity. Primary tumors from KPN (villinCreER Apcfl/fl; small intestine), KP (villinCreER KrasG12D/+ Trp53fl/fl TrgfbrIfl/fl; small intestine), AP (villinCreER Apcfl/+ Trp53fl/fl; small intestine) tumors were sampled without exclusion of submucosa or muscularis propia.

qRT-PCR
CDNA was generated by reverse transcription of the isolated RNA using the M-MuLV-Reverse Transcriptase kit (ThermoFisher-Scientific, 28025010) according to the manufacturer’s protocol. qPCR was performed using the DyNAmo HS SYBR Green qPCR kit.
IHC and Image Analysis

Macroscopic intestinal tumor or metastases were analyzed for size and number and tumor burden or metastases burden was calculated according to the manufacturer’s protocol. CT-values were normalized to β-Actin (Actb) CT-values. mRNA expression levels were calculated according to the ΔCT method and expressed as 2^(-ΔCT). Primers sequences can be found in Table S8.

Chromatin Immunoprecipitation

For chromatin immunoprecipitation (ChIP) the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, 9005) protocol was used according to the manufacturer’s instructions. In brief, KPN organoids were grown in medium conditions as described above. Cells were cross-linked for 10 minutes at room temperature and chromatin was fragmented by microcococcus nuclease following by three sonication cycles to generate DNA fragments. Incubation with RBPUSH (D10A4) XP® Rabbit mAb (Cell Signaling Technology, S513) or recommended concentration of rabbit normal IgG control (Cell Signaling Technology, 2729) for 16 hours at 4 °C was performed. The sequences of oligonucleotides used as qChIP primers are listed in Table S8.

Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin and processed by standard histology processing techniques. The following antibodies were used: CD3 (AbCam Ab16669, pH6 1:50), CD4 (eBioscience 14-9766-82, ER2 Leica, 1:500), CD8 (eBioscience 14-0808-82, ER2 Leica,1:500), S100A9 (Santa Cruz sc-8115, pH6 1:1000), Ly6G (clone 1A8, 2B Scientific BE0075-1, ER2 Leica, 1:60000), CD44 (BD Biosciences 550538, pH6, 1:250), SOX9 (Millipore AB5535, pH6, 1:500), β-catenin (BD Biosciences 610154, pH8, 1:50), MLH11 (Abcam ab92312, pH6, 1:200), CALD1 (Sigma HPA008066, ER2 Leica, 1:400), IGFBP7 (Sigma HPA002196, ER2 Leica, 1:100), pSMDA3 (Abcam Ab52903, pH6, 1:40), αSMA (Sigma-Aldrich A2547, pH6, 1:25000), N1ICD (D3B8 Cell Signaling Technology 4147, Protaqs IX, BioCyc, 401603692, 1:50).

To stain collagen or fibrin presence within tissue sections Picro Sirius Red staining technique was used. Briefly, de-waxed slides were immersed in Picro Sirius Red solution for 2 hours. Picro Sirius Red Solution: 0.1% Direct red 80 (Sigma, 41496LH) in distilled water and 0.1% Fast green FCF (Raymond Lamb, S142-2) in distilled water were mixed in equal volumes and then diluted 1:9 with Aqueous Picric acid solution. Post staining slides were dehydrated according to standard protocols and mounted for analysis.

Hypoxia was detected by administration of Hypoxyprobe (Hypoxyprobe HP1-100; 100 µl intraperitoneal) 1 hour before sampling and detected using Hypoxyprobe recognizing antibody (Hypoxyprobe HP1-100, pH6, 1:150).

In Situ Hybridisation

In situ hybridisation (ISH) analysis was performed using the RNAscope 2.5 LS kit (Brown, 322100) detection kit (Advanced Cell Diagnostics, Hayward, CA) on a Leica Bond Rx autostainer strictly according to the manufacturer’s instructions. Staining was performed on 4 µm formalin fixed paraffin sections which were cut and then placed in a 60 °C oven for 2 hours prior to staining. To ensure the quality and integrity of the available RNA the tissue being investigated was tested with the positive control probe (mm-Ppib, 313918). After probe quality control were the results evaluated. To further ensure accuracy and integrity of the staining a negative control probe (mm-DapB, 312038) was used to confirm that the tissue staining seen was accurate due to binding with the target probe and not non-specific. Probes: Cxcl1 (407728), Cxcl2 (437588), Cxcl3 (492758), Cxcl5 (487678), Cxcl9 (400338), Axin2 (400338), Lgr5 (312178), c-Myc (413458), Smad7 (428413), Tgfβ1 (400338), Tgfβ2 (406188), positive control probe Ppib (313918) and negative control probe DapB (312038).

RNA-Sequencing

The quality of the purified RNA was tested on an Agilent 2200 Tapestation using RNA screen tape. Libraries for cluster generation and DNA sequencing were prepared following an adapted method from the Illumina TruSeq RNA LT Kit. Quality and quantity of the DNA libraries was assessed on a Agilent 2200 Tapestation (D1000 screentape) and Qubit (Thermo Fisher Scientific) respectively. The libraries were run on the Illumina Next Seq 500 using the High Output 75 cycles kit (2x36cycles, paired end reads, single index). Quality checks on the raw RNA-Seq data files were done using fastqc version 0.11.2 and fastq_screen version 0.11.3. RNA-seq paired-end reads were aligned to the GRCh38 (Church et al., 2011) version of the mouse genome using tophat2 version 2.0.13 (Kim et al., 2013) with Bowtie version 2.2.4.0 (Langmead and Salzberg, 2012). Expression levels were determined and statistically analyzed by a combination of HTSeq version 0.6.1, the R environment, version 3.2.2, utilizing packages from the Bioconductor data analysis suite and differential gene expression analysis based on the negative binomial distribution using the DESeq2 (Anders and Huber, 2010). All RNA-sequencing data have been deposited in the ArrayExpress database under accession number E-MTAB-6363.

Tumor and Metastasis Scoring

Macroscopic intestinal tumor or metastases were analyzed for size and number and tumor burden or metastases burden was calculated as number of tumors times tumor size. All metastases were confirmed histologically.

Image Analysis

IHC and ISH (RNA-scope) images were digitalized using a SCN400F slide scanner (Leica Microsystems, Milton Keynes, UK) at 20x (IHC) or 40x (ISH) resolution. Scanned images were analyzed using HALO Image analysis software (V2.0.1145, Indica Labs). Tumors
were analyzed for the percentage of positive cells for N1ICD, CD3, CD4, CD8a, Ly6G and S100A9. Tumor areas were manually defined using the HALO software and scoring was performed in a blinded manner for all samples. β-catenin staining was analyzed manually and considered as positive when > 10% of the tumor area was strongly positive for nuclear β-catenin. For Cxcl5/Cxcr2 co-analysis serial sections (3.5 µm sections) were stained for Cxcl5 and Cxcr2 and scanned at 40x magnification. Slides were then automatically aligned utilizing the image registration module within the HALO package. Sequential, non-overlapping, paired fields of view were then individually scored.

**CRC Patient Data for In Silico Analysis**

CRC patient data were obtained from different public sources. Expression data and clinical/genetic annotation from the TCGA project (Cancer Genome Atlas Network, 2012) were downloaded from the FIREHOSE repository (https://gdac.broadinstitute.org/). This included RNA-seq expression data generated by the Illumina HiSeq (n=326) and Genome Analyzer (n=172) platforms (RSEM normalized data). After log transformation, data from both platforms were combined into a single dataset (n=498), by correcting platform-specific effects with the ComBat algorithm (Johnson et al., 2007) as implemented in the sva R package (Leek et al., 2012). From the NCBI GEO repository microarray expression data and clinical/genetic annotations for the following 11 datasets (total n=1981): GSE39582 (n=585), GSE13294 (n=294), GSE14333 (n=157), GSE17536 (n=177), GSE17537 (N=55), GSE20916 (n=81), GSE2109 (n=315), GSE23878 (n=35), GSE33113 (n=90), GSE35896 (n=62) and GSE37892 (n=130). The microarray data were normalized, summarized and log2 transformed using robust multiarray analysis (rma) and batch effects (both between and, where present, within dataset) were removed using Combat. After normalization the probe sets were annotated using the hg133plus2.db annotation R package (Carlson, 2017). In the case of multiple probe sets interrogating a specific gene, the probe set with the highest mean intensity was selected as representative for that gene. CMS labels for all datasets were obtained from Guinney et al. (Guinney et al., 2015) and CRIS labels were obtained from Isella et al. (Isella et al., 2017). A processed gene expression dataset of CRC liver metastases (E-TABM-1112, n=120) was obtained from ArrayExpress.

**Mouse Model and Patient Gene Expression Signatures**

Mouse model gene expression signatures were generated by quantifying differential gene expression data using the DESeq2 R package (Love et al., 2014). Patient-based CMS signatures were derived from the TCGA dataset where differential expression was determined using the limma R package (Ritchie et al., 2015). Human-to-mouse orthologue mappings were obtained from Biomart (http://biomart.org) (Smedley et al., 2015) using the interface provided by the biomartR R package (Durinck et al., 2009). In case of one-to-many human-to-mouse mappings the mapping with the highest homology percentage was selected. For the mouse model-patient correlation analysis 75 genes were selected that were the up-regulated most significantly (> 0.75 log fold change) in each of the four CMSs (300 genes total) and five CRISs (375 genes total) and calculated the Pearson correlation coefficient with the ComBat algorithm (Johnson et al., 2007) as implemented in the sva R package (Leek et al., 2012). From the NCBI GEO repository microarray expression data and clinical/genetic annotations for the following 11 datasets (total n=1981): GSE39582 (n=585), GSE13294 (n=294), GSE14333 (n=157), GSE17536 (n=177), GSE17537 (N=55), GSE20916 (n=81), GSE2109 (n=315), GSE23878 (n=35), GSE33113 (n=90), GSE35896 (n=62) and GSE37892 (n=130). The microarray data were normalized, summarized and log2 transformed using robust multiarray analysis (rma) and batch effects (both between and, where present, within dataset) were removed using Combat. After normalization the probe sets were annotated using the hg133plus2.db annotation R package (Carlson, 2017). In the case of multiple probe sets interrogating a specific gene, the probe set with the highest mean intensity was selected as representative for that gene. CMS labels for all datasets were obtained from Guinney et al. (Guinney et al., 2015) and CRIS labels were obtained from Isella et al. (Isella et al., 2017). A processed gene expression dataset of CRC liver metastases (E-TABM-1112, n=120) was obtained from ArrayExpress.

**Infiltration Scores**

Infiltration scores were calculated with the MCPcounter R package (Becth et al., 2016a, 2016b) and then standardised per cell type.

**KPN VS. APN Signature Survival Analysis**

KPN (n=9) vs. APN (n=3) tumor and organoid KPN (n=3) and APN (n=4) gene expression signatures were determined using the same procedure described in the previous paragraph. The 500 most significantly regulated genes were selected to construct the KPN vs. APN signature. No formal p value cut-off was used but all Benjamini-Hochberg adjusted p values were below 1x10^-6. TCGA and NCBI GEO expression datasets (excluding samples of tumor stage IV, without recurrence data or those without a CMS label n=1018) were combined into a single dataset correcting batch effect using ComBat. Expression values were mean-centred gene-wise and individual samples were scored by calculating the Pearson correlation coefficient with the KPN vs. APN signatures. Samples with a correlation score > 0.1 were assigned to the positive group, those with a correlation score < -0.1 to the negative group. Kaplan-Meier plots were generated using the survival R package and the survival distributions were compared with the log-rank test.

**GSEA**

Geneset enrichment analyses (GSEA) were run using the GSA R package with sample permutation (10,000 permutations) and gene standardization using all genes in the expression dataset, taking the unadjusted p values as output. The input genesets are listed in Table S5. The two TGF-β genesets used to assess TGF-β signaling activity in organoids where obtained from (Plasari et al., 2009; Calon et al., 2012).

**NOTCH-score**

The NOTCH-score was calculated by summing the standardized expression of a panel of NOTCH related genes (Kwon et al., 2016): JAG1, JAG2, DLL1, DLL3, DLL4, NOTCH1, NOTCH2, NOTCH3, NOTCH4, HES1, HES2, HEY1, HEY2 and DTX1. For the recurrence-free survival analysis of CRC patients from the TCGA dataset, patients were stratified using the within-group median NOTCH-score. KRAS mutants were called when mutations encoding amino acids G12, G13 and A143 were present. Kaplan-Meier plots were generated using the survival R package and the survival distributions were compared with the log-rank test.
WNT-score
The WNT-score was calculated by summing the standardised expression of a panel of WNT related genes: ASCL2, AXIN2, BMP4, MRT04, HILPDA, NOP16, KITLG, LGPR5, MYC, NOP2, PPIF, SOX4, PAAF1, ZIC2 & ZNRF3.

KPN/KP-score
A KPN (n=3) vs. KP (n=3) organoid gene signature was generated by quantifying differential gene expression data using the DESeq2 R package. We selected the 100 most significantly up-regulated genes (log fold change > 1). No formal p value cut-off was used but all Benjamini-Hochberg adjusted p values were below 1x10^{-2}. The KPN/KP-score was calculated by summing the standardised expression of the genes in the KPN vs. KP score (Table S7).

Human Serrated Signature
The human serrated signature was derived by comparing WNT target gene expression (Van der Flier et al., 2007) in serrated and tubular adenomas (GSE45270 (n=13) and GSE79460 (n=16)) (Fessler et al., 2016) using the limma R package. The microarray data were normalized summarized and log2 transformed using robust multi array analysis (rma) and batch effects were removed using Combat. After normalization the probe sets were annotated using the hgu133plus2.db annotation R package. In case of multiple probe sets interrogating a specific gene, the probe set with the highest mean intensity was selected as representative for that gene. The mouse model signatures were generated using the DESeq2 R package (Love et al., 2014).

Whole Genome Sequencing
Organoid (tumor derived) and tail DNA were extracted using DNAeasy kit (Qiagen, 69504) as per the manufacturer’s instructions. DNA concentration and quality were determined by Nanodrop spectrophotometry and by PicoGreen (Invitrogen, P11496). Whole genome sequencing was performed using 151bp paired-end sequencing on the Illumina HiSeq X platform. Short insert libraries were constructed using prepared flow cells, and clusters generated using standard methods. Samples were sequenced at an average depth of 39x with a minimum coverage of 26x. Data were mapped to the mouse reference genome (GRCm38) using the bwa-mem alignment tool (Li and Durbin, 2009). All whole genome sequencing data have been deposited in the European Nucleotide Archive (ENA) under ENA accession ID: ERP040713.

Somatic Mutation Detection
Somatic variants were detected using CaVEMan, an expectation maximization–based somatic substitution detection algorithm (Jones et al., 2016). Candidate somatic variants were then filtered for quality and to remove known mouse genome variations (Keane et al., 2011). Single point mutations overlapping known structural variants in any of the mouse genomes were also removed due to high misalignment rates in these regions. Small insertion and deletion (indel) detection was performed using the cgp-pindel pipeline (v0.2.4w) (Ye et al., 2009). Detected indels were then filtered for quality, sequence coverage in both tumor and normal, strand bias and for overlap with known simple repeats or indels in the in-house normal panel. Selected mutations were confirmed by Sanger sequencing.

Copy Number Detection
Tumor specific copy number changes were reported using Control free software (Boeva et al., 2012).

DATA AND CODE AVAILABILITY
The datasets generated during this study are available at the European Nucleotide Archive: ERP040713 and ArrayExpress: E-MTAB-6363. The codes supporting the current study have not been deposited in a public repository because of dependencies on in-house software and data infrastructure, but are available from the corresponding author on request.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed using GraphPad Prism software (v7.03 GraphPad software, La Jolla, CA, USA) and R (version 3.4.3) performing tests as indicated and were considered statistically significant, with * p<0.05, ** p<0.01, *** p<0.001, **** p<0.001.
Supplemental Information

Epithelial NOTCH Signaling Rewires the Tumor Microenvironment of Colorectal Cancer to Drive Poor-Prognosis Subtypes and Metastasis

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Figure S1 related to Figure 1. (A) NOTCH-score in TCGA CRC tumors separated by CMS. p value was calculated using a two sample two-tailed Welch t-test, comparing CMS4 to CMS1/2/3. The boxes indicate interquartile range (IQR), horizontal black lines median. The whiskers range to a maximum of 1.5 times the IQR. Data points outside 1.5 IQR are represented by individual dots. (B) Recurrence free survival (RFS) of CRC patients (TCGA), stratified using the NOTCH-score, all CMS stratified patients; blue line shows expression ≤ median score, red line shows expression > median score. (C) RFS of CRC patients (TCGA), stratified using the NOTCH-score, of all four CMSs in patients with KRAS mutations; blue line shows expression ≤ median score, red line shows expression > median score. (D) RFS of CRC patients (TCGA), stratified using the NOTCH-score, CMS4 stratified patients; blue line shows expression ≤ median score, red line shows expression > median score. (E) Left panel: Quantification of percentage positive or negative nuclear N1ICD expression (IHC) in human CRC liver metastases. Right panel: representative images of N1ICD expression (IHC) in human CRC liver metastases; scale bars, 200 µm.
Figure S2 related to Figure 1. (A) Left panel: Kaplan-Meier survival curves of intestinal tumor free survival of KPN mice or KPN mice homozygote for Rosa26<sup>N1</sup>icd. Right panel: Percent incidence of macroscopic metastases per genotype of villinCre<sup>ER</sup> Kras<sup>G12D/+</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1</sup>icd (KPN<sup>N1icd/N1icd</sup>) n=10, villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1</sup>icd (KPN<sup>N1icd/N1icd</sup>) n=29 mice. (B) Left panel: Kaplan-Meier survival curves of intestinal tumor free survival of KPN mice or KPN mice heterozygote for floxed Trp53, analyzed by log-rank (Mantel–Cox) test. Right panel: Percent incidence of macroscopic metastases per genotype of villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1</sup>icd (KPN<sup>N1icd/N1icd</sup>) n=6, villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1</sup>icd (KPN<sup>N1icd/N1icd</sup>) n=29 mice. (C) Left panel: Kaplan-Meier survival curves of intestinal tumor free survival of KPN mice or KPN mice with Trp53<sup>fl/fl</sup>R172H (KPfl/R172H) n=12, villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1</sup>icd (KPN<sup>N1icd/N1icd</sup>) n=29 mice. In (A-C) DIA = diaphragm, LN = Lymph-node, Peri = peritoneal carcinomatosis. Quantification of percentage of positive cells per crypt stained for (D) Olfm4, (E) Lgr5, (F) Lysozyme (Lyz) and (G) BrdU. WT = villinCre<sup>ER</sup>; K = villinCre<sup>ER</sup> Kras<sup>G12D/</sup>; P = villinCre<sup>ER</sup> Trp53<sup>fl/fl</sup>; N = villinCre<sup>ER</sup> Rosa26<sup>N1icd/+</sup>; PN = villinCre<sup>ER</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1icd/+</sup>; KN = villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Rosa26<sup>N1icd/+</sup>; KP = villinCre<sup>ER</sup> Kras<sup>G12D/</sup> Trp53<sup>fl/fl</sup> Rosa26<sup>N1icd/+</sup>. Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed.
Figure S3 related to Figure 2, 3 and 4. (A) Distribution of primary tumors in the intestines of mice with indicated genotypes. AP n=10; APN n=12; KP n=14; KPN n=28. (B) Representative IHC for the indicated markers on primary tumors from APN and KPN mice at endpoint; scale bars, 100 μm. (C) Representative IHC for the indicated markers on liver metastases of KPN mice at endpoint; scale bars, 100 μm. (D) Heat-map showing WNT ligand expression in primary tumors of intestinal GEMMs. (E) Quantification of primary tumor stage and percentage of mice with metastasis at day 85 after tamoxifen administration; KP n=5; KPN n=5. (F) Average KP organoid size or number seven days post single cell seeding under indicated conditions; LGK974 10 μM. Each sample was generated from a separate tumor n=3; error bars represent mean ± SEM.
Figure S4 related to Figure 3 and 4. (A) Landscape of somatic mutations in KPN tumor derived organoids; number of somatic point mutations per sample. (B) Nucleotide changes per sample. (C) Landscape of somatic copy number changes (bottom right panel). Number of gains and losses per sample (top panel). Absolute copy number profile (bottom left panel). Gain and loss frequency across different chromosomes. (D) GSEA results for organoids with indicated genotypes and CMS1-4 CRC patient tumors. Replicates: APN (organoids) n=4; KP (organoids) n=3; KPN (organoids) n=3. (E) Box plots showing standardized infiltration scores (calculated with MCPcounter) in primary tumor derived expression profiles for indicated genotypes. The boxes indicate interquartile range (IQR), horizontal black lines median. The whiskers range to a maximum of 1.5 times the IQR. Data points outside 1.5 IQR are represented by individual dots.
Figure S5 related to Figure 5. (A) Representative images from ISH of Cxcl1, Cxcl2 and Cxcl3 expression in KPN primary tumors; scale bars, 100 μm. (B) Representative image (top) and expression correlation analysis (bottom) of Cxcl5 and Cxcr2 expression (ISH) in KPN primary intestinal tumors per field of view (n=2 KPN primary tumors; 30 FOVs per tumor were analyzed); p value by Pearson correlation; scale bars, 100 μm. (C) Expression analysis of neutrophil markers encoding genes as indicated in CRC patient tumors (TCGA) per CMS. P values were calculated using the ANOVA-test. (D) Top: Neutrophil infiltration in CRC patient tumors (TCGA) per CMS; p=3x10^-8, CMS4 vs. others. Bottom: Expression analysis of Neutrophil infiltration in CRC patient tumors (TCGA) per CRIS; p=0.01, CRIS-B vs. others. P values were calculated using the ANOVA-test. (E) Recurrence free survival of patients with treatment naïve CRC metastases (ETABM-1112), stratified using the neutrophil infiltration-score. Blue line shows expression ≤ median score (low), red line shows expression > median score (high). (F) Cumulative survival of patients (Glasgow-cohort), stratified using IHC for MPO or CXCR2 in metastases post synchronous resection of colorectal primary tumors and liver metastases. P values were calculated by log-rank (Mantel-Cox) test. The boxes in C and D indicate interquartile range (IQR), horizontal black lines median. The whiskers range to a maximum of 1.5 times the IQR. Data points outside 1.5 IQR are represented by individual dots.
Figure S6 related to Figure 5. (A) Kaplan-Meier survival curves for intestinal tumor free survival of KPN mice treated with vehicle (n=13) or CXCR2 small molecule (CXCR2sm) (n=10), treatments started on day 85 after tamoxifen administration. (B) Tumor burden quantified in vehicle (n=10) and CXCR2sm (n=10) treated KPN mice at endpoint. (C) Blood neutrophil counts at endpoint for vehicle (n=6) and CXCR2sm (n=7) treated mice; error bars represent mean ± SEM, analyzed by Mann–Whitney U test, one-tailed. (D) Quantification of IHC for CD3 positive cells per KPN liver after one week treatment (n=5). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (E) Quantification of IHC analysis of indicated markers per primary tumor in KPN mice treated with vehicle or CXCR2sm from day 85 after tamoxifen administration (n≥5). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (F) Quantification of IHC analysis of indicated markers per liver of KPN mice treated with vehicle or CXCR2sm from day 85 after tamoxifen administration (n=5). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (G) Kaplan-Meier survival curves for intestinal tumor free survival of KPN mice treated with isotype control (2A3) (n=11) or Ly6G blocking antibody (1A8) (n=12). Treatment started 85 days after tamoxifen administration. (H) Tumor burden of 2A3 (n=10) or 1A8 (n=9) treated KPN mice at endpoint. (I) Blood neutrophil count at endpoint for indicated treatments (2A3 n=10, 1A8 n=8). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, one-tailed. (J) Quantification of IHC for CD3 positive cells per KPN liver after one week treatment (n=5). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (K) Quantification of IHC analysis of indicated markers per primary tumor of KPN mice treated with 2A3 or 1A8 from day 85 post tamoxifen administration (n=24). Error bars represent mean ± SEM. (L) Quantification of IHC analysis of indicated markers per liver of KPN mice treated with 2A3 or 1A8 from day 85 after tamoxifen administration (n=24). Error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed.
**Figure S7 related to Figure 6 and 7.**

(A) NOTCH-score expression in KP (n=3) and KPN (n=3) organoids. P value was calculated with a t-test. (B) KPN/KP-score in CRC patient tumors (TCGA) per CMS. P value was calculated using the ANOVA-test; p=5.31x10^-33, CMS4 vs. others. (C) Expression analysis of KPN/KP-score in CRC patient tumors (TCGA) per CRIS. P value was calculated using the ANOVA-test; p=0.00027, CRIS-B vs. others. (D) Correlation of the KPN/KP-score and neutrophil infiltration in human CRC patient tumors (TCGA). P value was calculated by Pearson correlation. (E) Correlation of the KPN/KP-score and neutrophil infiltration in human serrated adenoma. P value was calculated by Pearson correlation. (F) Recurrence free survival of patients with treatment naïve CRC metastases (E-TABM-1112), stratified using TGFβ2 expression. Blue line shows expression ≤ median score (low), red line shows expression > median score (high). (G) Expression analysis of TGFβ2 in CRC patient tumors (TCGA) per CMS. P value was calculated using the ANOVA-test; p=1x10^-4, CMS4 vs. others. (H) Expression analysis of TGFβ2 in CRC patient tumors (TCGA) per CRIS. P value was calculated using the ANOVA-test; p=0.01, CRIS-B vs. others. (I) Correlation of TGFβ2 expression and the NOTCH-score in CRC patient tumors (TCGA). P value was calculated by Pearson correlation. (J) Expression analysis of TGFβ2 in CRC patient tumors (TCGA) with high or low NOTCH-score. P value was calculated with a t-test. (K) Representative ISH analysis of Tgfb1 expression in KP primary tumor; scale bar, 100 μm. (L) Number and burden of macroscopic liver metastases four weeks post intra splenic transplantation of KP organoids (vehicle n=5, CXCR2sm n=5). (M) Quantification of flow cytometry analysis for neutrophils in blood (left) or liver metastases (right) four weeks post transplantation (vehicle n=5, CXCR2sm n=5), error bars represent mean ± SEM, analyzed by Mann–Whitney U test, one-tailed (N) Quantification of flow cytometry analysis for T cell subsets (% Granzyme B (GzB)+/CD8+) in liver metastases four weeks post transplantation (vehicle n=5, CXCR2sm n=5). (O) Quantification of flow cytometry analysis for T cell subsets (% Interferon-γ (IFN-γ)+/CD4+) in liver metastases four weeks post transplantation (vehicle n=5, CXCR2sm n=5). (P) Number of macroscopic liver metastases four weeks post intra splenic transplantation of KP organoids in immune-deficient nude mice (vehicle n=5, CXCR2sm n=5, Alk5i n=5). In S7L and S7N error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. The boxes in A, B, C, G, H and J indicate interquartile range (IQR), horizontal black lines median. The whiskers range to a maximum of 1.5 times the IQR. Data points outside 1.5 IQR are represented by individual dots.
Figure S8 related to Figure 8. (A) Tumor burden of KPN mice at end point with indicated treatments (vehicle from day 85, Alk5i from day 85 or day 130, 1D11 or 1D11 isotype control from day 85 after tamoxifen administration), error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (B) Blood neutrophil counts at endpoint of KPN mice treated as indicated in A; error bars represent mean ± SEM. (C) Quantification of IHC for CD3 positive cells per KPN liver at endpoint (n=3), treated as indicated in A. Error bars represent mean ± SEM. (D) Quantification of IHC for Ly6G, CD3, CD4, CD8, αSMA and Sirius red positive cells per KPN primary tumor at endpoint after treatment as indicated in A; error bars represent mean ± SEM, analyzed by Mann–Whitney U test, two-tailed. (E) Representative H&E images of a KPN GEMM colonic primary tumor at end point; scale bars, 100 μm. (F) Representative H&E images of intra colonic transplanted KPN organoids at end point; scale bars, 100 μm. (G) Kaplan-Meier survival curves of intestinal tumor free survival from mice with indicated genotypes (Alk5fl/fl n=14; Ly6Gcre Alk5fl/fl n=13) and KPN organoid transplantation.