COLORECTAL CANCER AND WNT SIGNALLING

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Colorectal cancer (CRC) is a heterogeneous disease comprising distinct molecular subgroups that differ in their histopathological features, prognosis, metastatic propensity, and response to therapy. Utilising state-of-the-art preclinical models harbouring key driver mutations, our group is interrogating the molecular mechanisms underpinning CRC. Our overarching goals are to identify early-stage diagnostic biomarkers and develop stage- and subtype-specific targeted therapies.

Most colorectal tumours develop from benign adenomas through the adenoma–carcinoma pathway, typically entailing aberrant activation of Wnt signalling, with loss-of-function mutations in the negative Wnt-regulator APC sufficient for adenoma formation. Progression to adenocarcinoma is underpinned by the accumulation of compounding mutations in oncogenic and tumour-suppressive pathways, including KRAS, TP53, and TGFβ. In this past year, we have developed tractable models of CRC ranging from early-stage adenomas through to treatment-refractory, *KRAS*-mutant CRCs, with *ex vivo* organoid cultures adding value to our suite of *in vivo* models.

The Wnt-antagonist NOTUM is a druggable mediator of cell competition in early-stage CRC

Widespread screening can detect tumours at early stages amenable to therapeutic intervention, rendering an urgent need for the validation of early-detection biomarkers and the identification of druggable targets to prevent progression of early-stage disease. We therefore sought to understand how common initiating mutations impact the dynamics of adenoma formation.

Given that the inactivation of the tumour suppressor APC is a frequent early event in adenoma initiation, we sought to identify how *Apc*-mutant intestinal stem cells (ISCs) compete with their wild-type neighbours to achieve clonal dominance and fixation (Flanagan *et al.*, 2021, *Nature*). Using gene expression profiling, we found that APC-deficient adenomas expressed an abundance of transcripts for several secreted Wnt antagonists, relative to APC-proficient tissues, with the most highly upregulated gene, *Notum*, encoding a secreted WNT deacylase that disrupted WNT ligand-binding (Figure 1A). Culture of wild-type organoids in conditioned medium, collected from *Apc*-mutant cells, curtailed growth (Figure 1B), decreased the expression of ISC-associated genes, and induced differentiation. Addition of a NOTUM inhibitor (Figure 1B), or genetic deletion of *Notum* in *Apc*-mutant organoids, abolished the effects of the conditioned medium.

In VilCre^{ER}*Apc*$^{Min/+}$ mice, genetic or pharmacological inhibition of NOTUM compromised the ability of *Apc*-mutant cells to expand and form intestinal adenomas, significantly prolonging survival (Figure 1C).

Deletion of *Notum* in *Apc*-mutant *Lgr5*-ISCs impaired their ability to outcompete wild-type counterparts. Interestingly, wild-type *Lgr5*-ISCs in the vicinity of *Apc*-mutant cells exhibited reduced expression of the WNT-regulated stemness marker SOX9, whereas cells adjacent to *Apc*-mutant *Notum*^{cKO} cells retained robust levels of SOX9, consistent with a role for secreted NOTUM in driving the differentiation of wild-type *Lgr5*-ISCs. Secreted NOTUM could therefore act in a paracrine fashion to inhibit Wnt signalling in neighbouring non-transformed wild-type ISCs, inducing their differentiation and withdrawal from the cell cycle, and ultimately driving their removal from the stem cell pool (Figure 1D). By contrast, WNT ligand-independent, APC-deficient, super-competitor cells could expand unabated with their progeny taking over the entire intestinal crypt.

Our findings identified NOTUM as a druggable mediator of cell competition and mutation fixation during the early stages of adenoma development. Bolstering the fitness of wild-type ISCs by inhibiting NOTUM might serve as a viable approach for preventing progression of early-stage disease in high-risk individuals with hereditary CRC.

Epithelial TGFβ/ALK5 engages growth-factor signalling to drive intestinal tumourigenesis with aggressive features

Building on our work with early-stage CRCs, we sought to develop means to identify so-called born-to-be-bad CRCs that are endowed with inherent metastatic potential, which enables metastasis-founder cells to disseminate before the primary tumour is clinically detectable. Indeed, histological assessment alone fell short of reliably identifying early-stage aggressive lesions destined to progress to metastatic spread. By transcriptionally profiling an early-stage human CRC cohort, enriched for born-to-be-bad lesions that went on to relapse, we correlated aggressive traits with elevated epithelial cell-intrinsic—rather than stromal—TGFβ signalling (Figures 2A–2C), alongside oncogenic *KRAS* mutations and APC deficiency (Flanagan *et al.*, 2022, *Nature Comms*).

We therefore generated VilCre^{ER}*Apc*$^{fl/+}$*Kras*$^{G12D/+}$*Alk5*^{CA} mice, where the *Alk5*CA allele encoded a constitutively active form of the TGFβ/ALK5 receptor, which instigated downstream TGFβ signalling in the intestinal epithelium. We found that, in the presence of concurrent *Apc* and *Kras* mutations, epithelial-specific activation of TGFβ signalling elicited rampant acceleration of intestinal tumourigenesis, engendering dissemination-prone tumours with born-to-be-bad transcriptomic features. Mechanistically, epithelial TGFβ signalling induced a growth-promoting EGFR-signalling module that synergised with mutant APC and KRAS to drive MAPK signalling, sensitising tumour cells to MEK and/or EGFR inhibitors and significantly prolonging survival (Figure 2D). Our data suggested that the convergence of activated Wnt, MAPK, and TGFβ/ALK5 signalling drove mitogenic and survival pathways that could be targeted therapeutically to slow the progression of intestinal tumours with aggressive behavioural traits.

Whereas tumour-suppressive roles are often ascribed to epithelial TGFβ signalling in CRC, our study found that epithelial cell-intrinsic TGFβ/ALK5 activation synergised with Wnt and MAPK signalling to drive intestinal tumourigenesis. Indeed, we identified epithelial TGFβ/ALK5 signalling as a potentially actionable, predictive biomarker in poor-prognosis, dissemination-prone early-stage CRCs that could reliably identify at-risk patients, offering an opportunity for early therapeutic intervention at a potentially curable stage. These findings were in line with the "Big Bang" model of CRC progression, which predicts that pro-invasive behaviour could be installed early in the disease trajectory. Overall, we identified epithelial TGFβ signalling both as a determinant of early dissemination and a potential therapeutic vulnerability of CRCs with born-to-be-bad traits.

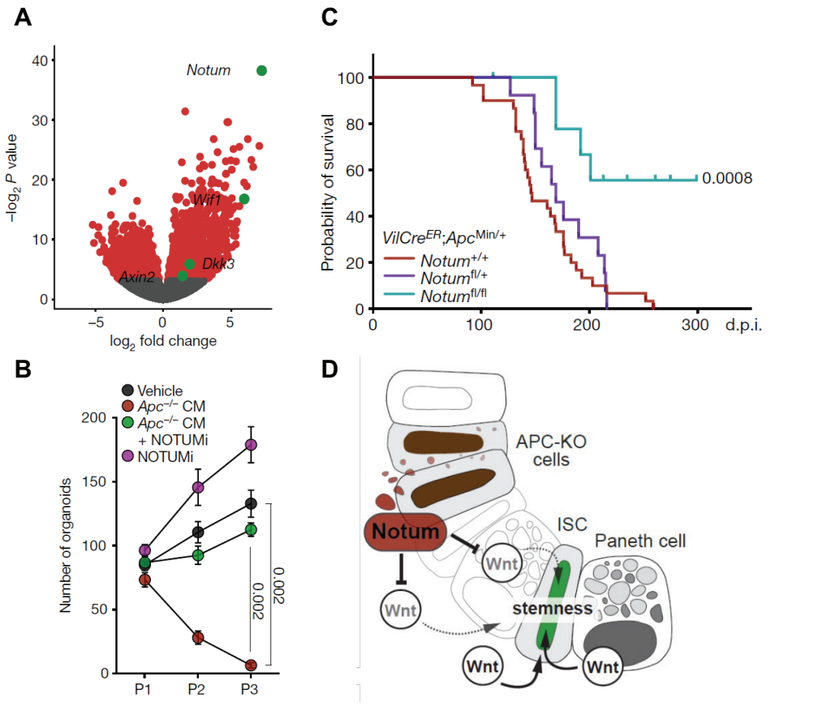

Figure 1
NOTUM is a prospective target for APC-deficient adenomas.
A Volcano plot showing genes differentially expressed between VilCre^{ER}*Apc*$^{fl/+}$ tumour tissue (n=5) and wild-type small intestine (n=3). Red, significantly altered genes; Green, Wnt antagonists. **B** Number of organoids formed over multiple passages (P1, P2, and P3) during culture in wild-type or *Apc*–/– conditioned medium (CM) supplemented with NOTUM inhibitor (NOTUMi). n = 6 mice per condition. **C** Survival of VilCre^{ER}*Apc*$^{Min/+}$*Notum*$^{+/+}$ (n=30), VilCre^{ER}*Apc*$^{Min/+}$*Notum*$^{fl/+}$ (n=13), and VilCre^{ER}*Apc*$^{Min/+}$*Notum*$^{fl/fl}$ (n=9) mice aged until clinical endpoint. **D** Schematic depicting the proposed model of NOTUM-mediated Wnt-pathway inhibition of wild-type ISCs (green) by *Apc*-mutant cells (brown). Curved arrows indicate activation; blunt-ended arrows indicate inhibition; dotted curved arrows indicate attenuation of Wnt activity.

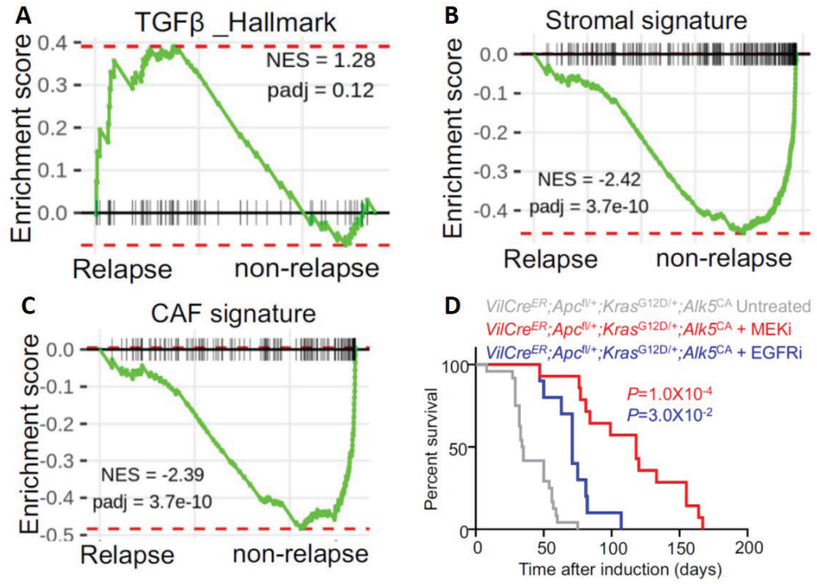

Figure 2
Epithelial TGFβ/ALK5 signalling—but not stromal content—correlates with relapsing, early-stage CRCs and sensitises to MAPK-targeted therapies.
A Hallmark gene set enrichment of TGFβ signalling in relapse cases compared with non-relapse samples. **B, C** Negative enrichment of cancer-associated fibroblast (CAF) (**B**) and stromal (**C**) signature gene sets in relapse cases compared with non-relapse samples. A–C were performed using fast gene set enrichment analysis. Benjamini–Hochberg FDR < 0.2. NES, normalised enrichment score; FDR, false discovery rate; padj, adjusted *P*-value (Benjamini–Hochberg multiple testing). **D** Kaplan–Meier survival curves for VilCre^{ER}*Apc*$^{fl/+}$*Kras*$^{G12D/+}$*Alk5*^{CA} mice treated daily with MEK1/2 inhibitor (MEKi) or EGFR inhibitor (EGFRi) and aged until clinical endpoint following tamoxifen induction. n=24 untreated (grey), n=14 MEKi (red), n=10 EGFRi (blue) mice. *P*=1.0 × 10^{-4} (MEKi), *P*=3.0 × 10^{-2} (EGFRi); log-rank (Mantel–Cox) test.

Kras allelic imbalance drives MAPK-dependent tumour initiation but sensitizes to MEK inhibition and fails to evoke metastasis

As mentioned above, oncogenic mutations in *Kras* lead to the constitutive activation of downstream effector pathways, including the MAPK-signalling cascade, and cooperate to drive colorectal tumourigenesis alongside loss of the tumour suppressor *Apc*. Oncogenic KRAS is strongly associated with therapy resistance, particularly to treatments targeting upstream or downstream signalling nodes such as EGFR, MEK, PI3K, and mTOR. Prevailing dogma holds that KRAS is a potent oncogene, with the gain of one mutant allele dominant over the remaining wild-type copy. As such, most studies to date have focused on the gain-of-function traits of oncogenic KRAS. However, accumulating evidence has argued for the existence of selective pressures that further augment oncogenic signalling through allelic imbalances that engender either focal amplifications of oncogenic *KRAS* or loss-of-heterozygosity at the wild-type allele. This implies that wild-type KRAS can influence the function of oncogenic KRAS. Yet, the role of wild-type KRAS, in the context of oncogenic KRAS, remains controversial with both pro- and anti-tumourigenic roles ascribed. We sought to better understand how wild-type KRAS impacts the fitness and drug responsiveness of CRCs, harbouring oncogenic KRAS, and to ascertain its impact on the tumour initiation and progression of *KRAS*-mutant tumours (Najumudeen *et al., in preparation*).

Towards this aim, we developed genetically engineered mouse models, which allowed the deletion of wild-type *Kras* in the context of oncogenic *Kras*^{G12D} in the phenotypically normal premalignant intestinal epithelium, the crypt-progenitor phenotype induced by acute *Apc* loss, long-term APC-deficient tumour development, and the metastatic setting. In the homeostatic small-intestinal epithelium, we found that mutant KRAS^{G12D} increased MAPK signalling, promoting enterocyte proliferation and suppressing Paneth-cell differentiation, with the deletion of the wild-type allele exacerbating these phenotypes and additionally increasing the abundance of secretory goblet cells, suggesting that wild-type KRAS restrains the activity of oncogenic KRAS^{G12D} in the premalignant setting. We further found that deletion of wild-type *Kras* potentiated oncogenic KRAS^{G12D} activity and downstream MAPK signalling, increasing the capacity of KRAS^{G12D}-mutant APC-deficient cells to dedifferentiate and initiate tumourigenesis, suggesting that wild-type KRAS functions as a tumour suppressor in the presence of oncogenic KRAS^{G12D}. In turn, however, this rendered tumours addicted to oncogenic KRAS signalling and conferred enhanced sensitivity to MEK inhibition, unveiling an exploitable therapeutic vulnerability (Figure 3A). Conversely, the presence of wild-type KRAS rendered KRAS^{G12D}-driven tumours resistant to MEK1/2 inhibition (Figure 3A) by dampening their dependence on MAPK signalling, posing a major clinical challenge. Importantly, deletion of wild-type *Kras* in oncogenic KRAS^{G12D}-driven, p53-mutant, aggressive tumours promoted initiation but significantly perturbed tumour progression and metastasis, reducing serrated morphological features, compromising invasiveness, and altering the tumour microenvironment. Furthermore, the loss of wild-type *Kras* significantly accelerated tumourigenesis and reduced survival (Figure 3B) in our aggressive NOTCH1-driven, KRAS-mutant intestinal adenocarcinoma model that metastasised to the liver (VilCre^{ER}*Kras*$^{+/G12D}$*Trp53*$^{fl/fl}$*Rosa26*$^{N1icd/+}$ and VilCre^{ER}*Kras*$^{fl/G12D}$*Trp53*$^{fl/fl}$*Rosa26*$^{N1icd/+}$ mice, designated KPN and KPN KF, respectively). Notably, however, loss of wild-type *Kras* in this model abrogated invasiveness and metastatic competence (Figure 3C). Molecularly, KPN KF tumours lacking wild-type KRAS exhibited significantly elevated Wnt-pathway activity and lacked expression of neutrophil chemoattractants (*Tgfβ2* and chemokines, such as *Cxcl1*, *Cxcl3*, and *Cxcl5*; Figure 3D) in their pre-metastatic niche, thereby blunting metastasis formation (Figures 3C and 3E). These studies provided new insights into KRAS biology and revealed a critical role for wild-type KRAS in the therapeutic resistance and metastatic proclivity of mutant KRAS-driven CRCs. These findings further suggested that, in addition to screening CRC-patients for *KRAS* mutation status, stratifying patients for *KRAS* allelic status might discern those who would derive benefit from inhibition of downstream effector signalling.

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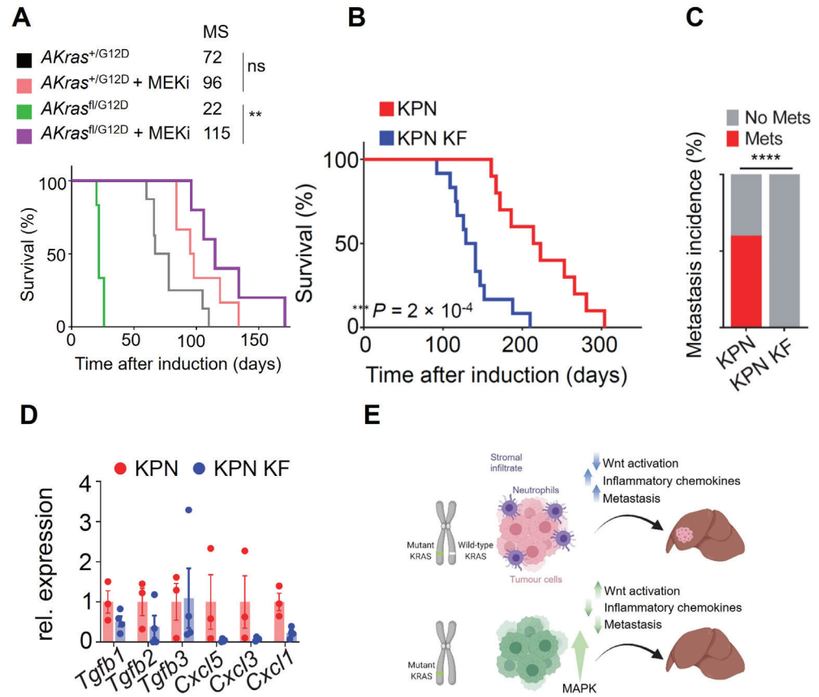

Figure 3
Loss of wild-type Kras increases sensitivity to MEK inhibition and suppresses the metastatic traits of *Kras*^{G12D} colorectal tumours.
A Kaplan–Meier survival curves for VilCre^{ER}*Apc*$^{fl/+}$*Kras*$^{+/G12D}$ (*AKras*$^{+/G12D}$) and VilCre^{ER}*Apc*$^{fl/+}$*Kras*$^{fl/G12D}$ (*AKras*$^{fl/G12D}$) mice, treated with MEK-inhibitor (MEKi) one day post tamoxifen-induction and aged until clinical endpoint. Median survival (MS) values are indicated. *Apc*$^{+/fl}$*Kras*$^{+/G12D}$, n=8; *Apc*$^{+/fl}$Kras$^{+/G12D}$ + MEKi, n=6; *Apc*$^{+/fl}$*Kras*$^{fl/G12D}$, n=6; *Apc*$^{+/fl}$*Kras*$^{fl/G12D}$ + MEKi, n=5.**P=0.0014, ns=not significant; log-rank (Mantel–Cox) test. **B** Kaplan–Meier survival curves for VilCre^{ER}*Kras*$^{+/G12D}$*Trp53*$^{fl/fl}$*Rosa26*$^{N1icd/+}$ (KPN) and VilCre^{ER}*Kras*$^{fl/G12D}$*Trp53*$^{fl/fl}$*Rosa26*$^{N1icd/+}$ (KPN KF) mice aged until clinical endpoint. KPN, n=10; KPN KF, n=12. Median survival (MS) values are indicated. ***P=2 × 10^{-4}; log-rank (Mantel–Cox) test. **C** Incidence of metastasis (%) in KPN and KPN KF mice aged until clinical endpoint. Median survival (MS) values are indicated. ****P< 0.0001. KPN, n=10; KPN KF, n=12. **D** Relative expression of transcripts encoding *Tgfβ* ligands and chemokines in organoids derived from KPN and KPN KF tumours. KPN, n=3; KPN KF, n=4. **E** Schematic depicting the mechanisms whereby loss of wild-type *Kras* activates Wnt signalling and reduces neutrophil recruitment, compromising the metastatic competence of KPN KF tumours.