Notum inhibits local Wnt signalling to increase the competitiveness of *Apc*-mutant clones

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The *APC* tumour suppressor gene is the most commonly mutated gene in colorectal cancer (CRC). Loss of *Apc* in intestinal stem cells (ISCs) drives aberrant Wnt signalling and adenoma formation in mice [1]. We previously showed that a reduction in WNT-ligand secretion increases the ability of *Apc*-mutant ISCs to colonise a crypt (fixation) and accelerate tumourigenesis [2]. Here, we investigate key mechanistic processes whereby *Apc*-mutant cells gain a clonal advantage over wild-type counterparts to achieve fixation. We find that *Apc*-mutant cells are enriched for transcripts encoding several secreted Wnt antagonists, with *Notum* being the most highly expressed. Indeed, conditioned medium from *Apc*-mutant cells suppresses the growth of wild-type organoids in a Notum-dependent manner. Furthermore, *Notum*-secreting mutant clones actively inhibit the proliferation of surrounding wild-type crypt cells and drive their differentiation, thereby outcompeting them from the niche. Importantly, genetic or pharmacological inhibition of Notum is sufficient to abrogate the expansion of *Apc*-mutant cells and their ability to form intestinal adenomas. Taken together, we demonstrate *Notum* as a key mediator during the early stages of mutation fixation, which can be targeted to restore wild-type cell competition and thus, offer novel preventative strategies for high-risk patients.

Main

The colonic epithelium displays one of the highest mutation rates of all tissues [3,4], with loss-of-function mutations in the *APC* tumour suppressor considered a key early event in colorectal cancer (CRC) initiation [5]. For a mutation to be maintained within a crypt, it needs to become "fixed", by mutant cells outcompeting wild-type intestinal stem cells (ISC) from the crypt [6,7]. Previous studies revealed that *Apc* loss (or *Kras* activation) confer a clonal advantage to ISCs [7,8], increasing their probability of fixation/winning within the crypt and, in the case of *Apc* mutation, driving adenoma formation. Even though APC-deficient clones have an increased probability of "winning", they can still be stochastically eliminated from the ISC pool i.e. lose. This suggests uncovering the molecular mechanisms by which APC-deficient cells outcompete wild-type cells could lead to novel chemo-preventative approaches.

APC is a negative regulator of Wnt signalling that functions as an integral part of the destruction complex, which directs the phosphorylation and degradation of β-catenin [9]. Since *Apc*-mutant tumours exhibit constitutive Wnt-pathway activation, we first sought to identify genes differentially upregulated in *Apc*-mutant cells relative to the normal intestinal epithelium. For this, we performed transcriptomic analysis of tumours that develop in *VillinCre^{ER};Apc$^{fl/+}$* (hereafter *VilCre^{ER};Apc$^{fl/+}$*) mice following the sporadic loss of the remaining copy of *Apc* [10], akin to human CRC [11]. As expected, Wnt-target genes were highly upregulated in these *Apc*-mutant tumours (Extended Data Fig. 1a). The most highly upregulated gene was *Notum* (Fig. 1a), which encodes a secreted WNT deacylase that disrupts WNT-ligand binding to Frizzled receptors [12]. We have previously shown that Notum expression in the ISC niche increases with age, and secreted Notum can impair self-renewal and tissue regeneration of wild-type ISCs during ageing [13]. Therefore, to characterise the expression of Notum specifically in wild-type and *Apc*-mutant ISCs, we utilised the *Lgr5-EGFP-IRES-CreERT2* mouse model, which drives the expression of GFP from the Lgr5 locus [14]. In support of the tumour transcriptomic analysis (Fig. 1a), FACS isolated *Apc*-mutant (hereafter *Lgr5Cre^{ER};Apc$^{fl/fl}$*) Lgr5^{+} cells show

significant enrichment for *Notum* expression compared to wild-type Lgr5^{+} cells (*Lgr5Cre^{ER};Apc$^{+/+}$*) already seven days post tamoxifen-induction (Fig. 1b). To confirm elevated *Notum* expression in cells with Wnt-activating mutations, we performed *in situ* hybridisation for *Notum* (*Notum*-ISH) across a number of our Wnt-driven tumour models (Extended Data Fig. 1b). In line with our previous work, expression of *Notum* in young (3-6 month old) wild-type animals was minimal (Fig. 1c), whilst all three tumour models (*Lgr5Cre^{ER};Ctnnb1$^{Ex3/+}$*, *Apc$^{1322T/+}$*, and *Apc$^{Min/+}$*) exhibited pronounced *Notum* expression demarcating the tumour epithelium. Furthermore, the *Notum* expression pattern mirrored nuclear β-catenin staining in serial sections from tumour tissues (Fig. 1d), positioning *Notum* as a faithful surrogate marker of *Apc*-mutant clones. To formally address the relationship between *Apc* mutation status and *Notum* expression, we performed ISH on serial sections from *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice 10 days following tamoxifen-induction. We found that *Notum* expression accurately stained the emerging clones harbouring the targeted deletion of *Apc* and maintained robust *Notum* expression in the developing adenoma epithelium over time (Extended Data Fig. 1c, d) [2]. We have previously shown that *Apc*-mutant cells are refractory to WNT-ligand inhibitors *in vivo* [2], but that age-induced expression of *Notum* can inhibit Wnt signalling in wild-type ISCs [13]. Moreover, *Apc*-mutant cells act as super-competitors in the developing fly, and eliminate neighbouring cells in a Notum-dependent manner [15]. Jointly, these data suggest that emerging *Apc*-mutant clones may deploy paracrine effectors, such as Notum, to actively inhibit essential Wnt signalling in neighbouring wild-type ISCs.

To functionally test whether *Apc*-mutant cells can suppress their wild-type neighbours via secreted factors, we grew wild-type small intestinal organoids in conditioned medium (CM) collected from organoids derived from *VilCre^{ER};Apc$^{+/+}$* (wild-type; hereafter WT CM) and *VilCre^{ER};Apc$^{fl/fl}$* (*Apc*-mutant; hereafter *Apc$^{-/-}$* CM) mice (Fig. 1e). The growth and viability of wild-type organoids cultured in *Apc$^{-/-}$* CM was profoundly inhibited (Fig. 1f, g and Extended Data Fig. 1e). Functionally, this corresponded to a striking reduction in organoid number (Fig.

1h) over time, strongly suggesting that *Apc*$^{-/-}$ CM inhibited stemness. Consistent with this notion, these organoids exhibited a dramatic decrease in the expression of Wnt-target genes and ISC markers and, conversely, an increased expression of differentiation markers (Fig. 1i), reminiscent of the decrease in stemness caused by age-associated induction of *Notum* [13]. Complementary to our data, the accompanying manuscript by van Neerven *et al*. describes co-culture experiments between wild-type and *Apc*-mutant organoids, whereupon *Apc*-mutant cells rapidly out-compete wild-type counterparts, dominating the co-culture. Moreover, these authors observed comparable effects when wild-type organoids were cultured in *Apc*$^{-/-}$ CM, which is consistent with our observations. Together, these findings underscore the importance of paracrine interactions between neighbouring competing cells and suggest that factors, secreted by *Apc*-mutant cells, can impair the stemness of wild-type ISCs by suppressing their capacity for growth and promoting their differentiation.

We next assayed the growth kinetics of wild-type organoids cultured in WT or *Apc*$^{-/-}$ CM, supplemented with a selective Notum inhibitor (10nM LP-92056, hereafter NOTUMi) [16,17]. Interestingly, the addition of NOTUMi rescued the growth-arrest phenotypes observed in wild-type organoids treated with *Apc*$^{-/-}$ CM (Fig. 1g). Remarkably, by P3, the number of organoids in cultures grown in *Apc*$^{-/-}$ CM containing NOTUMi had increased to levels comparable to those observed in WT CM (Fig. 1h). Importantly, upon NOTUMi-supplementation, also the expression of Wnt-target genes and markers for ISCs and differentiation in *Apc*$^{-/-}$ CM-treated organoids was brought to a level comparable with WT CM (Fig. 1i). However, *Lgr5* expression was notably higher after NOTUMi in both *Apc*$^{-/-}$ CM and WT CM, demonstrating the potency of NOTUMi and suggesting that even modest *Notum* expression can impact ISCs. To confirm the robustness of Notum-mediated ISC suppression, we interbred *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$ mice with mice carrying a conditional *Notum* allele [18] (hereafter *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$;*Notum*$^{fl/fl}$ mice) and collected the conditioned medium from tamoxifen-induced *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$;*Notum*$^{fl/fl}$ organoids (*Apc*$^{-/-}$;*Notum*$^{-/-}$ CM) (Extended Data Fig. 1f, g). Importantly, wild-type organoids

treated with *Apc*$^{-/-}$;*Notum*$^{-/-}$ CM had comparable growth kinetics to vehicle control, which was reversed/blocked in cells treated with *Apc*$^{-/-}$;*Notum*$^{-/-}$ CM supplemented with recombinant Notum (Fig. 1j, Extended Data Fig. 1g).

In addition to *Notum*, several other Wnt inhibitors were also upregulated in the transcriptomic analysis of *Apc*-mutant cells compared to wild-type and confirmed by ISH (Fig. 1a, Extended Data Fig. 2a, b). To test whether these genes are also implicated in the competitive advantage of *Apc*-mutant cells, wild-type organoids were treated with recombinant Wif1 and/or Dkk3 and assayed for changes to morphology and growth (Extended Data Fig. 2c). Interestingly, compared to Notum-treated cells, which potently suppressed organoid expansion, Wif1 and Dkk3 failed to perturb organoid growth when added individually or in combination to wild-type cells, despite being upregulated in *Lgr5Cre*^{ER};*Apc*$^{fl/fl}$;*Notum*$^{fl/fl}$ organoids (Extended Data Fig 2c, d). Additionally, although all tested secreted WNT inhibitors (Notum, Wif1 and Dkk3) reduced Wnt-target gene expression, Notum displayed the greatest suppression of the pathway, supporting its ability to block wild-type organoid growth (Extended Data Fig. 2e). Of note, recombinant Notum, Wif1, Dkk3 or NOTUMi had no effect on the growth of *Apc*-mutant organoids (Extended Data Fig. 2f, g), indicating Notum preferentially suppresses the growth of wild-type rather than *Apc*-mutant cells. Taken together, these findings indicate that Notum is both sufficient and necessary to inhibit wild-type organoid expansion, and is the key paracrine negative regulator of Wnt signalling produced by *Apc*-mutant cells to inhibit wild-type stem cell function.

To investigate whether the observed organoid phenotype translates to intra-cryptal competition *in vivo*, we administered low-level tamoxifen induction (0.15 mg) to clonally induce *Apc* and *Notum* deletion in only occasional *Lgr5*$^{+}$ cells per crypt. As the *Apc* locus does not recombine at the same frequency with the widely used *Rosa26* reporter [2], we used nuclear β-catenin staining as a surrogate to measure *Apc*-mutant clonal outgrowth in the presence or

absence of *Notum* (Fig. 2a). To ensure accurate clonal quantification, we scored only β-catenin^{+}/*Notum*$^{+}$ clones in *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{+/+}$ mice and β-catenin^{+}/*Notum*$^{-}$ clones in *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{fl/fl}$ mice. Furthermore, we measured the relative ratio of fully-to-partially fixed crypts [2] and the total number of mutant (β-catenin^{+}) clones emerging over time (Fig. 2b, c). In *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{+/+}$ mice, *Apc*-mutant clones became rapidly fixed so that, by 14 days post induction, over 50% of lesions were fixed within a crypt (Fig. 2b). In striking contrast, only 10–15% of double mutant *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{fl/fl}$ clones were fixed at this timepoint (Fig. 2b), consistent with the observed reduction in the total number of double mutant clones in *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{fl/fl}$ mice over time (Fig. 2c). Next, we evaluated the size distribution of *Apc*-mutant clones at the crypt base over time, as detected by nuclear β-catenin^{+}/lysozyme^{+} staining (Fig. 2d). Compared to *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{+/+}$ mice, which showed fully clonal crypts from 21 days post-tamoxifen, clones from *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{fl/fl}$ mice failed to expand at the same rate, indicative of impaired clone fixation following *Notum* deletion (Fig. 2e). Collectively, these data show that in the early phases of clonal competition, Notum-mediated Wnt inhibition is essential for *Apc*-mutant cells to win over neighbouring wild-type cells and fix the crypt with mutant progeny.

We next asked whether co-deletion of *Apc* and *Notum* in *Lgr5*$^{+}$ ISCs could affect long-term clonal expansion and tumour formation. Unlike our observations at earlier time points, we observed no difference in the survival of *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$*;Notum*$^{fl/fl}$ mice or their overall tumour burden compared to controls (Extended Data Fig. 3a–c). However, the majority of the large adenomas that grew out had escaped *Notum* deletion and expressed robust levels of *Notum* (Extended Data Fig. 3d), suggesting that retaining Notum function confers a survival/clonal advantage during adenoma development and, conversely, that Notum loss-of-function bestows a clonal disadvantage upon *Apc*-mutant cells. To further validate our findings, we generated a novel Notum conditional knockout allele (termed *Notum*^{c} to distinguish between *Notum*^{fl} mice) by inserting two loxP sites flanking exon 8 (Extended Data Fig. 4a, b) and

crossed them to *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice (*Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{c/c}$*) (Extended Data Fig. 5a). Importantly, using this novel mouse model we observed robust recombination of *Notum* in *Apc*-mutant cells, which again translated to a reduction in fully-clonal *Apc*-mutant crypts and an overall decrease in small-, but not large-, intestinal *Apc*-mutant (β-catenin^{+}) clones following *Notum* deletion (hereafter *Notum*^{cKO}) (Extended Data Fig. 5b-f). Together with previous results (Fig. 2), this underscores a key requirement of Notum for *Apc*-mutant clonal fixation. However, *Notum* did not change the frequency or proliferation of *Apc*-mutant Lgr5^{+} cells, indicating that the reduced multiplicity of emergent mutant clones observed in *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{c/c}$* mice was indeed due to changes in early ISC clonal competition (WT vs *Apc*-mutant) and not tumour intrinsic mechanisms (Extended Data Fig. 5g). Of note, *Notum* deletion did not cause any gross alterations to intestinal homeostasis (Extended Data Fig. 4c-e), but did render Lgr5^{+} ISCs cells more clonogenic when cultured in vitro (Extended Data Fig. 4f, g), consistent with its function as a lowly expressed mTOR-responsive Wnt regulator [13].

Our in vitro findings suggest Notum inhibits wild-type ISCs by decreasing stemness and promoting their differentiation, and therefore, we next sought to determine whether the same processes were responsible for facilitating *Apc*-mutant fixation in vivo. To test this, we quantified the frequency of EdU^{+} wild-type crypt cells within *Apc*-mutant crypts, with and without *Notum* (*Notum*^{WT} vs *Notum*^{cKO} respectively) following high-dose tamoxifen (3 mg) (Fig. 3a). Consistent with our earlier results (Fig. 1), wild-type cells residing within *Notum*^{WT}-mutant crypts (*Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{+/+}$*) had reduced proliferation compared to wild-type cells three-crypt diameters away from a mutant clone (Fig. 3a). In striking contrast, wild-type cells cohabiting *Notum*^{cKO}-mutant crypts (*Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{c/c}$*) showed similar proliferation to distant wild-type crypts. In order to assess the cellular target most influenced by Notum-mediated Wnt inhibition, we next measured the relative nuclear expression of Wnt-regulated Sox9 [19,20] in ISCs (adjoining Paneth cells) and in transit-amplifying (TA) progenitor cells (those not in contact with Paneth cells) in *Apc*-mutant crypts and the surrounding wild-type epithelium

(Fig. 3b). Importantly, the expression of Sox9 in wild-type ISCs was most dramatically reduced in crypts containing *Apc*-mutant clones and was closer to the level detected in TA cells, suggestive of a partial differentiation and loss of stemness (Fig. 3b). However, the expression of Sox9 in wild-type ISCs either sharing the crypt with a *Notum*^{cKO}-mutant clone or located in the neighbouring crypts was not changed. These data suggest that Notum promotes the expansion of *Apc*-mutant clones by driving the differentiation of wild-type ISCs, which is consistent with its function in vitro (Fig. 1). Indeed, the number of Muc2^{+} secretory cells was increased in *Apc*-mutant crypts compared to *Apc*/*Notum*-mutant crypts (Fig. 3c). In support, van Neerven et al. demonstrate crypts neighbouring *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$ tumours show increased differentiation (see accompanying manuscript by van Neerven *et al*.). To examine whether *Apc*-mutant cells eliminate wild-type cells via apoptosis, as is the case in *Drosophila* [15], we measured the number of apoptotic cells within *Apc*-mutant clones and the neighbouring wild-type epithelium and found similar levels, independent of *Notum* status (Extended Data Fig. 6a). Moreover, *VillinCre*^{ER}*;Bax*$^{fl/fl}$;*Bak*$^{-/-}$ intestinal organoids (thereby deleting two key downstream pro-apoptotic BCL-2 family members, BAX and BAK) experienced robust organoid atrophy following treatment with Notum-proficient *Apc*$^{-/-}$ CM, which was rescued with the addition of NOTUMi (Fig. 3d), suggesting that Notum drives elimination of wild-type ISCs by differentiation rather than directly inducing apoptosis.

Interestingly, we observe the effects of Notum extend beyond the source of its production (*Apc*-mutant crypts), i.e Notum can influence the proliferation and differentiation of wild-type ISCs in crypts immediately adjacent to *Apc*-mutant clones (Fig. 3a, c). This is mirrored in the diminished size of wild-type crypts immediately adjacent to *Apc*-mutant clones (as measured by lateral cross-sectional area) compared to those at least two-crypt diameters away (Remote) (Fig. 3e). However, wild-type crypts adjacent to *Apc*/*Notum*-mutant crypts were of comparable size to their remote counterparts (Fig. 3e), indicating Notum can function over inter-cryptal distances. Collectively, these data confirm our conditioned media experiments and

demonstrate Notum promotes the expansion and fixation of *Apc*-mutant clones by directly suppressing WNT signalling and proliferation of neighbouring wild-type ISCs, while promoting their differentiation (Fig. 3a-c).

To mitigate the strong negative selection against *Notum*-deficient clones during adenoma formation observed using the *Lgr5Cre*^{ER} mouse, we crossed *Notum*$^{fl/fl}$ mice to *VillinCre*^{ER};*Apc*$^{Min/+}$ mice (hereafter *VilCre*^{ER};*Apc*$^{Min/+}$), which develop tumours following spontaneous loss-of-heterozygosity (LOH) of the remaining *Apc* allele [11]. Therefore, the fact that *Notum* is not expressed in the young intestine and *Apc*$^{Min/+}$ mice develop tumours from 60 days, we reasoned that deleting *Notum* prior to tumour formation would remove the strong selection against *Notum* deficient cells. To do this, we administered tamoxifen (2 mg) at 6 and 8 weeks of age to ensure robust deletion of *Notum* throughout the epithelium. In contrast to *VilCre*^{ER};*Apc*$^{Min/+}$;*Notum*$^{+/+}$ controls, *VilCre*^{ER};*Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ mice had a striking extension in survival due to reduced intestinal tumour burden (Fig. 4a), which was mirrored in *VilCre*^{ER};*Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ mice harvested at time point (85 days), prior to the onset of clinical symptoms (Fig. 4b, c). Interestingly, we observed a mixture of *Notum*$^{+}$ and *Notum*$^{-}$ adenomas in *VilCre*^{ER};*Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ mice (Fig. 4d, Extended Data Fig. 7a) which is consistent with the recombination efficiency of *VilCre*^{ER} in *Apc*-mutant tumour epithelia (Extended Data Fig. 7b) and a strong selection against *Notum*-deficient cells during early tumour formation. We also noted increased *Wif1*, but not *Dkk3*, expression in areas of tumour epithelium that remained *Notum*$^{-}$, suggestive of an attempt by *Apc*-mutant cells to upregulate other WNT inhibitors to supress neighbouring wild-type cells in the absence of Notum (Extended Data Fig. 7c). This is consistent with earlier observations (Extended Data Fig. 3) that demonstrate Notum is the primary mediator driving the fixation of *Apc*-mutant clones.

In the accompanying manuscript, van Neerven and colleagues investigate the role of the Wnt agonist LiCl in reducing the competitive fitness of *Apc*-mutant cells. Here, we undertook an

alternative approach to assess whether pharmacological intervention of Notum expression could yield therapeutic efficacy in our intestinal tumour models. To this end, *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice received daily treatment with NOTUMi (30 mg/kg) following low-dose (0.15 mg) tamoxifen-induction and sampled 21 days post tamoxifen. Encouragingly, NOTUMi treatment significantly reduced *Apc*-mutant fixation and the overall number of β-catenin^{+} lesions compared to vehicle treated mice (Fig. 4e-g, Extended Data Fig. 8a, b), recapitulating/mirroring our genetic models, and thus positioning Notum as a novel therapeutic target for *Apc*-driven intestinal tumours. Indeed, these data align well with our previous work that demonstrate the use of inhibitors targeting the O-acyltransferase Porcupine (Porcn), weaken the competitiveness of wild-type ISCs and accelerate *Apc*-mutant clonal fixation and subsequent tumourigenesis [2].

We have recently described how the differential expression of specific Wnt-target genes can be exploited to stratify human CRC into ligand -dependent (RSPO-fusion/RNF43) and –independent (APC) subtypes, which can inform suitability for WNT-targeted therapies [21]. As such, we confirmed strong expression of *NOTUM* in polyp and carcinoma samples from two independent patient cohorts (including patients with familial adenomatous polyposis (FAP)), but not in known RSPO-fusion mutant tumour epithelium or normal mucosa [21] (Fig. 4h, i and Extended Data Fig. 8c, d). To model ligand-dependent WNT transformation and assess potential *Notum* deregulation, we deleted the E3 ligases *Rnf43* and *Znrf3*, which function as negative feedback regulators of WNT signalling (Hao and Koo 2012), from the intestinal epithelium. Importantly unlike following loss of *Apc*, we did not observe expression of *Notum* (or Wif1 and Dkk3, not shown) in *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$* mice 14 days following tamoxifen induction, despite almost all epithelial cells expressing nuclear β-catenin (Extended Data Fig. 8e). More importantly, the lack of functional *Notum* in *Rnf43*/*Znrf3* mutant epithelia translated to no alterations in wild-type organoid growth following treatment with conditioned medium harvested from tamoxifen-induced *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$* organoids (*R*/*Z*$^{-/-}$ CM), and was

only perturbed following the addition of recombinant Notum (Extended Data Fig. 8f). These data confirm the mutational route taken to activate WNT signalling in CRC (*APC* vs *RSPO* or *RNF43*) deploy distinct transcriptional outputs that impose different molecular mechanisms to achieve mutant-clone fixation in the intestine.

Cell competition was first identified in the fly, whereby cells harbouring elevated Myc levels behave as so-called super-competitors that hyper-proliferate and dominate the competition by inducing the elimination of their Myc-low neighbours [22]. Following the onset of high Wg signalling in the fly, Notum is sufficient to eradicate surrounding cells [15]. Moreover, in the normal intestine, Paneth cell secreted Notum can reduce self-renewal, number and function of adjacent ISCs [13]. Here, we show that *Apc*-mutant cells hijack these competition processes and use Notum to attenuate the local Wnt environment and competitive fitness of neighbouring ISCs, thereby ensuring the dominance and retention of *Apc*-mutant progeny within the crypt. Importantly, *NOTUM* expression is significantly enriched in WNT ligand-independent human intestinal tumours, in contrast to ligand-dependent subgroups where *NOTUM* is silenced by methylation [21]. Together, this supports a model whereby paracrine Notum signalling is deployed to suppress the stemness of neighbouring wild-type ISCs, while not impacting the growth of WNT ligand-independent *Apc*-mutant cells (Fig. 4j). Finally, given multiple NOTUM inhibitors are currently under development, the robust pre-clinical data provided in the currently study argues that NOTUM inhibition could provide an efficacious strategy for preventing clonal fixation and mutant expansion underpinning adenoma development, thereby extending survival [17].

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Methods

Mouse studies

All animal experiments were performed in accordance with UK Home Office regulations (under project license 70/8646) and were subject to review by the Animal Welfare and Ethical Review Board of the University of Glasgow and Finnish National Animal Experimentation Board. All mice were maintained on a mixed C57BL/6 background. Mice of either gender, aged 2-6 months, were induced with a single intraperitoneal (i.p) injection of 0.15, 2 or 3 mg tamoxifen as indicated (Sigma-Aldrich, #T5648). For *Lgr5Cre*^{ER};*Notum*$^{c/c}$ studies, mice were fed tamoxifen food (Harlan, #TD55125) for 10 days and analysed 4 or 8 months after. The transgenes/alleles used for this study were as follows: *VilCre*^{ER} [23], *Lgr5-EGFP-IRES-Cre*^{ER} (designated *Lgr5Cre*^{ER}) [14], *Apc*^{580S} [24], *Apc*^{Min} [11], *Apc*^{1322T} [25], *Notum*^{fl} [18], *Ctnnb1*^{Ex3} [26], *Rnf43*^{fl} [27], *Znrf3*^{fl} [27] and *Bax*^{fl}/*Bak*^{ko} [28]. For tumour growth studies, mice were aged until they showed clinical signs of intestinal disease (anaemia, hunching, and/or weight loss). For Notum inhibition studies mice were dosed with LP-95032/NOTUMi (30 mg/kg, twice daily oral gavage) or equivalent volume of vehicle (distilled water+0.5% Tween-20).

Generation of *Notum* conditional allele

The *Notum* conditional loss-of-function allele (*Notum*^{c}) was generated using genOway customised mouse model services. Briefly, a homology fragment for the C57BL/6 mouse Notum gene locus was isolated and a targeting vector containing loxP sites flanking *Notum* exon 8 and an FRT-flanked neomycin selection cassette was generated. The NheI linearised targeting vector was electroporated into C57BL/6 mouse embryonic stem (ES) cells followed by G418 selection. Correctly targeted ES cell clones were identified by PCR and Southern blot analysis (Extended Data Fig. 4b). Validated ES cell clones were injected into blastocysts for the generation of chimeras. Viable chimeras were crossed with C57BL/6 Flp deleter mice to remove the neomycin cassette and generate mice with the desired conditional *Notum*^{c} allele (Extended Data Fig. 4a). Upon Cre-mediated deletion, *Notum* exon 8 is lost causing a

frameshift leading to a premature stop codon in exon 9 and destroying the catalytic triad required for Notum pectin acetylesterase (PAE) activity.

Patient material

Formalin-fixed paraffin embedded intestinal polyps and carcinoma tissue was collected from anonymised patients who had undergone curative surgery and completion of adjuvant therapy for stage III CRC (Victor Trial study; PMID: 20837956, REC reference: 17/NW/0252).

Organoid culture

Mouse small intestinal crypts were isolated from wild-type, *Lgr5Cre^{ER};Notum$^{c/c}$*, *VilCre^{ER};Bax$^{fl/fl}$;Bak$^{-/-}$*, *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$*, *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* and *VilCre^{ER};Apc$^{fl/fl}$* mice as previously described [29]. Isolated crypts were resuspended in Matrigel (BD Bioscience), plated in 6-well plates, and overlaid with ENR growth medium comprising (Advanced DMEM/F12 (#12634010) supplemented with penicillin-streptomycin (#15070063), 10 mM HEPES (#15630056), 2 mM glutamine (#25030081), N2 (#17502048), B27 (# 17504044) (all from Gibco, Life Technologies), 100 ng/ml Noggin (#250-38), 500 ng/ml R-Spondin (#315-32), and 50 ng/ml EGF (#315-09) (all from PeproTech). Conditioned medium (CM), derived from wild-type (WT CM), *VilCre^{ER};Apc$^{fl/fl}$* (*Apc$^{-/-}$* CM), *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* (*Apc$^{-/-}$;Notum$^{-/-}$* CM), *VilCre^{ER};Bax$^{fl/fl}$;Bak^{KO}* (*Bax.Bak$^{-/-}$* CM) and *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$* (*R/Z$^{-/-}$* CM) organoids, was collected over the course of 5 days, centrifuged at 1500 rpm for 5 min, and supplemented with the growth factors EGF, Noggin, and R-Spondin before use. Notum inhibition was achieved *in vitro* by adding 10 nM Lex44 (Lexicon Pharmaceuticals) [17] to the CM, and was replenished every other day. Recombinant Notum (1 μg/ml; R&D Systems, #9150-NO-050), Wif1 (1 μg/ml; R&D Systems, #135-CW-050) and Dkk3 (1 μg/ml; R&D Systems, #1118-DK-050) was mixed with standard growth medium and replenished every other day. For organoid growth rate experiments, ~100 wild-type organoids were resuspended in Matrigel and plated into 96-well plates. Following treatment, organoids were isolated, passaged and replated into 96-well plates. Organoids were quantified 3-5 days following passage. For clonogenicity experiments, 1,000 isolated Lgr5^{hi} cells were plated into 60%

Matrigel and overlaid with ENR medium containing 10 μM Chir99021 (GSK3 inhibitor; Tocris, #4423) and 10 μM Y27632 (ROCK inhibitor; Tocris, #1245) and changed after 2 days. Medium was replaced to regular ENR after 5 days of culture and colony numbers were quantified on day 7 after plating.

Organoid viability

Organoid viability was measured as previously described [30]. Briefly, organoids were mechanically disrupted and resuspended in TrypLE™ Express (Thermo Fisher Scientific, #12604013) with 100–200 U DNAse (Sigma, #4716728001) for 1 h at 37 °C. Cells were passed through a 40-μm strainer, counted, and seeded in 100 μl Matrigel/PBS (1:1 mixture) in a 96-well plate and overlaid with standard growth medium or CM plus treatments 48 h after seeding (described above). Viability was quantified using CellTiter-Blue® (Promega, #G8080) at indicated timepoints in combination with morphological grading/scoring of organoids with clear central lumen. The number of crypts/organoids was scored from a total of 30-50 organoids from 3 representative images per condition in n=2 independent experiments.

Single-cell sorting and analysis

Single cells from small intestinal crypts were isolated and analysed with flow cytometry as described in [13].

Clonal analysis and adenoma scoring

Small intestinal tissue was isolated, flushed with water, cut longitudinally and fixed in 10% neutral buffered formalin overnight at 4°C (β-catenin clonal scoring) or room temperature (*en face* scoring). For *en face* processing, small (~ 2 x 2 cm) pieces of tissue were paraffin embedded *en face* and cut into 5 μm sections. En face sections were stained and imaged using a Zeiss 710 confocal or an Opera Phenix high-content imaging platform (Perkin Elmer). For β-catenin IHC clonal analysis, the following criteria were used measure the ratio of fully fixed to partially fixed crypts and the relative percentage of clonal crypts; full - both walls of the crypt nuclear β-catenin^{+} and clones in contact with a Paneth cell; partial - one wall of the crypt

nuclear β-catenin^{+} and clones in contact with a Paneth cell; non-base - Nuclear β-catenin^{+} clone/s within crypt, but not in contact with a Paneth cell; undefined: Nuclear β-catenin^{+} clone/s not directly observable within main epithelial chain. The distribution and size of mutant clones and intestinal crypts was analysed and calculated using Harmony and Image J software respectively. Intestinal lesions were scored using the following criteria; microadenoma – small cluster of dysplastic cells contained within single crypt; lesion – 2 or more fully dysplastic crypts contained within a villus structure; small adenoma – collection of 2-5 connected fully dysplastic crypts that extend into the villus; large adenoma – fully dysplastic glands occupying >5 gland diameters and extend above villus.

RNA isolation and qPCR

RNA isolation was performed using the RNeasy mini kit (Qiagen, #74104) or TRIzol reagent (Thermo Fisher Scientific) for the sorted cells and cDNA was synthesised using high capacity cDNA reverse transcription kit, (#4368813) or for sorted cells RevertAid First Strand cDNA synthesis kit (#K1622) both (Thermo Fisher Scientific). SYBR Green (Thermo Fisher Scientific, #F410XL) qPCR reactions were performed with the BioRad system under standard conditions. Relative fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All $^{\Delta\Delta}$Ct values were normalised to housekeeping gene *Gapdh*. Primers used for qPCR: *Gapdh* forward, 5′-GAAGGCCGGGGCCCACTTGA-3′; *Gapdh* reverse, 5′-CTGGGTGGCAGTGATGGCATGG-3′; *Axin2* forward, 5′-GCGACGCACTGACCGACGAT-3′; *Axin2* reverse, 5′-GCAGGCGGTGGGTTCTCGGA-3′; *Lgr5* forward, 5′-GACAATGCTCTCACAGAC-3′; *Lgr5* reverse, 5′-GGAGTGGATTCTATTATTATGG-3′; *Notum* forward, 5′-CTGCGTGGTACACTCAAGGA-3′; *Notum* reverse, 5′-CCGTCCAATAGCTCCGTATG-3′; *Ascl2* forward, 5′-CTACTCGTCGGAGGAAAG-3′; *Ascl2* reverse, 5′-ACTAGACAGCATGGGTAAG-3′; *Lyz1* forward, 5′-GAGACCGAAGCACCGACTATG-3′; *Lyz1* reverse, 5′-CGGTTTTGACATTGTGTTCGC-3′; *Krt20* forward, 5′-AGTTTTCACCGAAGTCTGAGTTC-3′; *Krt20* reverse, 5′-GTAGCTCATTACGGCTTTGGAG-3′. *Wif1* forward, 5′-TCTGGAGCATCCTACCTTGC-3′;

Wif1 reverse, 5′-ATGAGCACTCTAGCCTGATGG-3′. *Dkk3* forward, 5′-CTCGGGGGTATTTTGCTGTGT-3′; *Dkk3* reverse, 5′-TCCTCCTGAGGGTAGTTGAGA-3′.

RNA sequencing and analysis

Whole tissue from the small intestine was used for RNA purification. RNA integrity was analysed with a NanoChip (Agilent RNA 6000 Nanokit #5067-1511). A total of 2 µg of RNA was purified via poly(A) selection. The libraries were run on an Illumina NextSeq 500 sequencing system using the High-Output kit (75 cycles). Analysis of RNAseq data was performed as previously described [31].

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) analysis was performed using the GSEA v2.0 software (Broad Institute) [32]. The comparison gene sets were obtained from publicly available datasets on the Broad Institute website [33].

RNA *in situ* hybridisation

In situ hybridisation for *Notum* (#472548), *Wif1* (#412368), *Dkk3* (#400938), *Lgr5* (#312178), and human *NOTUM* (#430311) mRNA (all from Advanced Cell Diagnostics) was performed using RNAscope 2.5 LS Reagent Kit–BROWN (Advanced Cell Diagnostics) on a BOND RX autostainer (Leica) according to the manufacturer's instructions. BaseScope (Advanced Cell Diagnostics) Apc^{Ex14} (Advanced Cell Diagnostics, #701641) was used to identify cells exhibiting Cre-mediated deletion of the wild-type *Apc* allele according to the manufacturer's instructions. Positive control probes (*Mm-Ppib*; Advanced Cell Diagnostics, #313918) were included in each run to ensure RNA integrity and staining specificity.

Immunohistochemistry and immunofluorescence

Intestines were flushed with water, cut open longitudinally, pinned out onto silicone plates and fixed in 10% neutral buffered formalin overnight at 4°C. Fixed tissue was rolled from proximal to distal end into swiss-rolls and processed for paraffin embedding. Tissue blocks were cut into 5 µm sections and stained with haematoxylin and eosin (H&E). Immunohistochemistry

(IHC) and immunofluorescence (IF) were performed on formalin-fixed intestinal sections according to standard staining protocols. Primary antibodies used for IHC and IF were against: β-catenin (1:100; BD Biosciences, #610154), Lysozyme (1:500; Dako, #A0099), RFP (1:100; Rockland, #600-401-379), Click-iT EdU Cell Proliferation Kit for Imaging Alexa 647 (Invitrogen, #C10340), Sox9 (1:200; Chemicon, #AB5535), Muc2 (1:300; Santa Cruz, #sc-15334), WGA (1 ug/ml; Invitrogen, #W32464), Cleaved Caspase3 (Asp175) (1:300; CST, #9661), GFP (1:300; abcam, #13970) and Ki67 (1:300; abcam, #15580). Representative images are shown for each staining.

Statistical analyses

The smallest sample size that could give a significant difference was chosen in accordance with the 3Rs. Given the robust phenotype of the *Apc*$^{fl/fl}$ mice and assuming no overlap in the control versus the experimental group, the minimum sample size was 3 animals per group. For analysis of organoid cultures, investigators were blinded when possible. Investigators performed all histological quantification blinded to genotype or treatment. Statistical analysis was performed with GraphPad Prism v8.0.0 for Windows (GraphPad Software) using two-tailed Mann–Whitney U-tests unless otherwise stated. Statistical comparisons of survival data were performed using the log-rank (Mantel-Cox) test. For individual value plots, data are displayed as mean ± standard error of the mean (s.e.m.). P-values ≤ 0.05 were considered significant. Statistical tests and corresponding P-values are indicated in the figure legends and figures respectively.

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Author contributions D.J.F, N.P, P.K and O.J.S designed and interpreted the results of all experiments. D.J.F, N.P, K.L, A.R, L.M, J.E, A.W, S.S, N.N, E.G, E.M, M.C.H and R.A.R performed all experiments and analysed the results. D.J.F performed and analysed organoid experiments. K.G, K.K and W.C processed and analysed the RNA-seq data. C.N performed ISH. N.S provided manuscript preparation advice. A.K.N, N.W, B.R, C.P, A.C.W, H.C, P.V.F, M.L, K.A, A.R, S.L, E.Y.J and J.P.V provided advice and reagents. D.J.F, N.S, N.P, P.K and O.J.S wrote the paper.

Competing interests The authors have no competing interests to declare.

Additional information

Supplementary Information is available for this paper

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Figure 1. *Apc*-mutant cells impair the growth of wild-type ISCs via Notum.

a, Volcano plot showing log2 fold change (x-axis) and –log2-transformed p-value (y-axis) of genes differentially expressed between *Apc*-mutant (*VilCre^{ER};Apc$^{fl/+}$*) tumour tissue and wild-type small intestine. Significantly altered genes are indicated in red, with negative regulators of Wnt signalling highlighted in green (n=3 WT mice, n=5 *VilCre^{ER};Apc$^{fl/+}$*mice). **b**, *Notum* expressed in Lgr5GFP^{hi} cells isolated from wild-type (*Lgr5Cre^{ER};Apc$^{+/+}$*; WT) and *Apc*-mutant intestines (*Lgr5Cre^{ER};Apc$^{fl/fl}$*; KO) 5-7 days following tamoxifen. **c**, *Notum*-ISH shows minimal expression of *Notum* in wild-type (3-5 month-old *Lgr5Cre^{ER};Apc$^{+/+}$*) small intestinal epithelium. Scale bar, 100 μm. **d**, Serial sections of intestinal tumour epithelium from *Lgr5Cre^{ER};Apc$^{fl/fl}$* (3 month-old) mice stained for *Notum* RNA (ISH) and β-catenin (IHC). Scale bar, 200 μm. **e**, Schematic illustrating experimental pipeline for wild-type (WT) organoid treatments. **f,** WT small intestinal organoids grown for 5 days in conditioned medium (CM) collected from wild-type (WT) or *Apc*-mutant (*Apc$^{-/-}$*) organoids. Organoids supplemented with selective NOTUM inhibitor (NOTUMi) rescues WT organoid growth in the presence of *Apc$^{-/-}$* CM. Organoids were derived independently from n=6 mice. Red arrowheads indicate organoid atrophy/death. Scale bar, 100 μm. **g**, Quantification of the number of crypt domains per organoid, n=6 biological replicates. **h**, Quantification of the number of organoids, formed over multiple passages (P1, P2, and P3), during culture in WT or *Apc$^{-/-}$* CM supplemented with NOTUMi. n=6 biological replicates per condition. **i**, qPCR for WNT-target genes (*Notum***,** *Axin2*), ISC markers (*Lgr5, Ascl2*), and cell-lineage markers (*Lyz1, Krt20*) expressed in organoids described in **f**. **j**, Quantification of WT organoids, formed over multiple passages (P1, P2, and P3), during culture in *Apc$^{-/-}$;Notum$^{-/-}$* CM supplemented with recombinant Notum. n=3 biological replicates per condition. Mann–Whitney one-tailed U-test. Representative images of organoids taken from 3 independent batches of organoids. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Extended Data Figure 1. Notum secreted by *Apc*-mutant clones inhibits wild-type organoid growth.

a, Volcano plot of significantly differentially expressed WNT-target genes (red dots) in *Apc*-mutant (*VilCre^{ER};Apc$^{fl/+}$*) tumour tissue compared to wild-type small intestine (n=3 WT mice, n=5 *VilCre^{ER};Apc$^{fl/+}$*mice). This is the same dataset as in Fig. 1a but with different genes highlighted (green dots). **b**, *Notum*-ISH shows high levels of *Notum* expression in multiple Wnt-driven tumour models of the indicated genotypes (*Ctnnb1$^{Ex3/+}$* – 30 days, *Apc$^{1322T/+}$* - 98 days, *Apc$^{Min/+}$* - 125 days). Scale bar, 200 μm. **c**, ISH of serial en face sections of intestinal tissue from *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice 10 days post tamoxifen-induction. BaseScope ISH for recombined *Apc* (*Apc^{Ex14}*-ISH) and *Notum* (*Notum*-ISH) shows exclusive expression of *Notum* in cells that have recombined *Apc* (cells lacking pink RNA dots in right panel). Boxed areas show close-up of Notum^{+} *Apc*-mutant crypts, demarcated by dashed line. Scale bar, 20 μm. **d**, *Notum*-ISH time course (days 6, 28, 60 and >100) post tamoxifen induction in *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice shows specific *Notum* expression in progressively dysplastic epithelium, Scale bar, 100 μm. **e**, Relative WT organoid viability measured at passage 3 following treatments as indicated. n=6 mice/condition. **f**, Schematic illustrating mouse crossing scheme to generate *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* mice and treatment of WT organoids with *Apc$^{-/-}$;Notum$^{-/-}$* conditioned medium (*Apc$^{-/-}$;Notum$^{-/-}$* CM). **g**, Representative images of WT organoids grown in *Apc$^{-/-}$;Notum$^{-/-}$* CM for 5 days and supplemented with recombinant Notum. Scale bar, 200 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. *P*<0.05 is considered significant.

Extended Data Figure 2. *Apc*-mutant cells upregulate negative regulators of Wnt signalling.

a, Raw sequence reads for transcripts encoding WNT-negative regulators, *Wif1* and *Dkk3*, in *Apc*-mutant (*VilCre^{ER};Apc$^{fl/+}$*) tumour tissue compared to wild-type (WT) small intestine (n=3 WT mice, n=5 *VilCre^{ER};Apc$^{fl/+}$*mice). **b**, Representative ISH for *Wif1* and *Dkk3* in *Lgr5Cre^{ER};Apc$^{fl/fl}$* tumour (top panels) and WT small intestine (bottom panels). n=3 mice. Images of mice shown aged between 3-4 months. Boxed areas show close-up of WT crypts. Scale bar, 200 μm. **c**, Quantification and representative images of WT organoids, formed over multiple passages (P1, P2, and P3), during culture supplemented with recombinant Wif1, Dkk3 and Notum. n=3 biological replicates per condition. Mann–Whitney one-tailed U-test. Scale bar, 200 μm. **d**, qPCR for WNT antagonists expressed by tamoxifen-induced *Lgr5Cre^{ER};Apc$^{fl/fl}$* (*Notum$^{+/+}$*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* (*Notum$^{fl/fl}$*) small intestinal organoids. n=4 biological replicates. **e,** qPCR for WNT targets expressed in WT organoids 3 days following treatment with indicated recombinant WNT antagonists. n=3 biological replicates. Mann–Whitney one-tailed U-test. **f**, Quantification of *VilCre^{ER};Apc$^{fl/fl}$* organoids 3 days after culture with recombinant Wif1, Dkk3 and Notum. n=3 biological replicates per condition. **g**, Relative organoid viability and representative images of *VilCre^{ER};Apc$^{fl/fl}$* intestinal organoids treated with vehicle or NOTUMi for 3 days. n=3 mice/condition. Scale bar, 100 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Figure 2. Notum is required for *Apc*-mutant fixation *in vivo*.

a, β-catenin IHC reveals fully and partially *Apc*-mutant fixed crypts in *Lgr5Cre^{ER};Apc$^{fl/fl}$* (*Notum$^{+/+}$*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* (*Notum$^{fl/fl}$*) mice. Scale bar, 200 μm. **b**, Ratio of fully-to-partially fixed crypts in *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice induced with 0.15 mg tamoxifen and sampled at 10, 14, and 21 days post induction (d.p.i). n=4 mice/genotype for each timepoint. **c**, Total number of nuclear β-catenin^{+} lesions in *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice over 10, 14, and 21 days post induction. n=4 mice/genotype for each timepoint. **d**, Representative whole mount β-catenin/Lysozyme immunofluorescence of *Apc*-mutant clones (red) from *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice 21 days post induction. Nuclei labelled with DAPI. Scale bar, 200 μm. **e**, Heat maps depict the relative frequency of mutant clones of the indicated size (columns) at various time points (rows) for both *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice. Graph displays the average clone size of *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice over time post tamoxifen induction. n=4 mice/genotype for each timepoint. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Extended Data Figure 3. Notum is required for *Apc*-mutant cells to form intestinal tumours.

a, Survival plot for *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{+/+}$* (*Notum$^{+/+}$*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* (*Notum$^{fl/fl}$*) mice aged until clinical endpoint following induction with 0.15 mg tamoxifen. (n=10 *Notum$^{+/+}$* mice, n=10 *Notum$^{fl/fl}$* mice). p=0.12, log-rank test. **b**, Total small intestinal tumour burden (area) per mouse from mice in **a**, (n=10 *Notum$^{+/+}$* mice, n=9 *Notum$^{fl/fl}$* mice). **c**, Small intestinal tumour number per mouse from mice in **a**, (n=10 *Notum$^{+/+}$* mice, n=10 *Notum$^{fl/fl}$* mice). **d**, Representative H&E and *Notum*-ISH staining on serial sections from *Notum$^{+/+}$* and *Notum$^{fl/fl}$* mice in **a**. Asterisks denote intestinal adenomas. Boxed areas are close-up of adenomas stained for *Notum*. Note that adenomas grow out as *Notum*-positive lesions in *Notum$^{fl/fl}$* mice suggesting that retaining *Notum* confers a survival advantage during adenoma development. Scale bars, 200 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Extended Data Figure 4. Generation and characterisation of novel Notum conditional knockout allele.

a, Schematic representing endogenous wild-type *Notum* locus and recombined *Notum*^{c} allele with the relevant genome editing sites indicated. **b**, Southern blot analysis of embryonic stem (ES) cell *Notum*^{c} clones and wild-type genomic DNA showing successful recombination at the *Notum* locus (4.7 kb product). The 13.6- and 4.7 kb bands represent endogenous and recombined alleles, respectively (arrows). **c**, Schematic of the tamoxifen (TMX) treatment regimen and analysis of tissues from *Lgr5Cre*^{ER}*;Notum*$^{c/c}$ mice. Representative agarose gel electrophoresis of products from conventional PCR detecting alleles for non-recombined and recombined *Notum*^{c} (238 and 513bp respectively) in *Lgr*5^{hi} cells isolated from *Lgr5Cre*^{ER};*Notum*$^{c/c}$ mice with or without tamoxifen induction. **d**, H&E staining of WT (*Notum*^{WT}) and *Notum*^{cKO} tissue harvested 8 months post-tamoxifen induction. Right panels shows regular crypt-villus architecture in both cohorts. Scale bar, 5mm. **e**, Cellular frequencies of crypt cells, analysed by flow cytometry, remain unchanged after *Notum* deletion (n=5 WT, n=6 *Notum*^{cKO}). ISCs (Lgr5^{hi}), transit-amplifying cells (Lgr5^{med} and Lgr5^{lo}), Paneth cells and Enteroendocrine cells (Endo). **f**, Representative images and quantification of organoid regeneration (shown as number of crypt domains per organoid) of WT and *Notum*^{cKO} (cKO) organoid cultures, (n=3 WT, n=4 *Notum*^{cKO}). Scale bar, 100 μm. **g**, Clonogenic growth of isolated Lgr5^{hi} cells is increased in *Notum*^{cKO} (cKO) compared to WT. Colonies were quantified 7 days post seeding, n=3 mice per group. Scale bar, 100 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant. Representative images taken at day 6 (**f**) and 7 (**g**) of culture.

Extended Data Figure 5. Notum is required for *Apc*-mutant cells to form intestinal tumours.

a, Schematic illustrating tamoxifen treatment regimen and tissue analysis days post induction (d.p.i.) from *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{+/+}$* (*Notum^{WT}*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{c/c}$* (*Notum^{cKO}*) mice. Representative images of small intestinal sections stained for β-catenin from *Notum^{WT}* and *Notum^{cKO}* mice 14 days following induction with 120 mg/kg (3 mg) tamoxifen. Arrows indicate dysplastic crypts with nuclear β-catenin (magenta border). Boxed area shows close-up of β-catenin^{+} crypts. Scale bar, 50 μm. **b**, Representative agarose gel electrophoresis of products from conventional PCR measuring relative recombination of *Apc^{fl}* and *Notum^{c}* alleles 5 days post tamoxifen induction. The 250- and 513 bp bands represent recombined *Apc* and *Notum* respectively. n=3 biological replicates. **c**, Quantification of β-catenin^{+} adenomatous foci per small intestine in *Notum^{WT}* and *Notum^{cKO}* mice induced with 3 mg tamoxifen and sampled at 7 and 14 d.p.i. n=4 for each genotype 7 d.p.i; n=18 *Notum^{WT}* mice, n=12 *Notum^{cKO}* mice 14 d.p.i. **d**, Quantification of β-catenin^{+} adenomatous foci per large intestine 14 d.p.i (n=18 *Notum^{WT}* mice, n=12 *Notum^{cKO}* mice). **e**, Representative images of β-catenin IHC depicting fully and partially fixed *Apc*-mutant crypts in *Notum^{WT}* and *Notum^{cKO}* mice, respectively. Mice were induced with 3 mg tamoxifen and sampled at 14 d.p.i (n=4 for each genotype). Ratio of fully-to-partially fixed crypts in *Notum^{WT}* and *Notum^{cKO}* mice. Scale bar, 100μm. **f**, Relative percentages of clonal crypt classification (clonal crypt phenotype) from mice described in **e**. **g**, Representative confocal images (left panel), quantification of Lgr5^{+} cell frequency within β-catenin^{+} *Apc*-mutant clones (middle plot) and proliferation of Lgr5^{+} and Lgr5^{-} *Apc*-mutant cells (β-catenin^{+}) of *Notum^{WT}* and *Notum^{cKO}* adenomas 14 d.p.i. Ki67 (magenta), Lgr5-EGFP (green), nuclei (cyan), β-catenin (white), adenomas (yellow dashed line). n=5 mice/genotype. Scale bar, 20 μM. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Figure 3. Notum inhibits cell proliferation and drives the differentiation of wild-type ISCs.

a, Representative images and quantification of wild-type crypt proliferation, marked by EdU incorporation (red) in *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{+/+}$* (*Notum^{WT}*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{c/c}$* (*Notum^{cKO}*) mice 14 d.p.i. Distance refers to the location of analysed wild-type crypt relative to the closest β-catenin positive clones (white, dashed yellow line). "0" refers to wild-type cells within the *Apc*-mutant crypt. Proliferation is represented in relation to distant crypts, more than three crypts away from *Apc*-mutant clones. n=5 for each genotype. Scale bar, 50 μm. **b**, Representative confocal images showing nuclear Sox9 expression (mean fluorescence intensity, MFI) in ISCs (green arrows) and transit-amplifying (TA) cells (blue arrows) next to an *Apc*-mutant clone (yellow border). Analysed nuclear region is excluding the cytoplasmic background seen in secretory cells (white asterisk). ISCs identified as cells adjoining WGA^{+} Paneth cells (red cell/left panel) and TA cells as not adjacent to WGA^{+} Paneth cell (blue arrows). Nuclei labelled with DAPI (white). Analysis of ISC nuclear Sox9 intensity is shown in relation to TA cells and with the same distance parameters as described in **a**. n=4 *Notum^{WT}* and n=5 *Notum^{cKO}*. Scale bar, 20 μm. **c,** Analysis of Muc2^{+} (green) positive cells in animals described in **a**. Scoring of positively labelled cells in clonal crypts was performed as described in **a.** n=5 *Notum^{WT}*, n=4 *Notum^{cKO}* mice. Scale bar, 50 μm. **d,** Quantification of tamoxifen induced *VilCre^{ER};Bax$^{fl/fl}$;Bak$^{-/-}$* organoids (*Bax$^{fl/fl}$;Bak$^{-/-}$*) following treatment with *Apc$^{-/-}$* CM ± NOTUMi. Representative images of *Bax$^{fl/fl}$;Bak$^{-/-}$* organoids taken 5 days following corresponding treatments. Organoids were derived independently from n=3 mice. Scale bar, 100 μm. **e**, Representative β-catenin immunofluorescence and quantification of wild-type crypt size adjacent to or remote from *Apc*-mutant clones (red) in whole mount small intestine from *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{+/+}$* (*Notum$^{+/+}$*) and *Lgr5Cre^{ER};Apc$^{fl/fl}$;Notum$^{fl/fl}$* (*Notum$^{fl/fl}$*) mice 14 d.p.i. (3 mg tamoxifen induction). Nuclei are labelled with DAPI (white). n=4 *Notum$^{+/+}$* and n=3 *Notum$^{fl/fl}$* mice. Scale bar, 50 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Figure 4. Inhibition of Notum limits *Apc*-mutant fixation and intestinal tumour progression.

a, Survival plot for *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{+/+}$, *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{fl/+}$ and *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ mice aged until clinical endpoint following induction with 2 mg tamoxifen at 6 and 8 weeks of age. (n=30 *Notum*$^{+/+}$, n=13 *Notum*$^{fl/+}$, n=9 *Notum*$^{fl/fl}$ mice, where 5 still alive at the time of submission. Of note, animal sampled at 101 days was tumour-free (censored)). log-rank test. **b**, **c**, Total tumour number (**b**) and burden (**c**) per mouse of genotypes shown. Mice were sampled at 85 days of age. n=11 *Notum*$^{+/+}$, n=8 *Notum*$^{fl/fl}$ mice. **d**, *Notum*-ISH on small intestinal tissue from mice described in **b.** Scale bar, 10 mm. **e**, Representative β-catenin immunofluorescence (red) of *Lgr5Cre*^{ER}*;Apc*$^{fl/fl}$ mice treated daily with vehicle or NOTUMi (30 mg/kg) following 0.15 mg tamoxifen induction and sampled 21 days post induction. Nuclei labelled with DAPI (white). Scale bar, 50 μm. **f**, Ratio of fully-to-partially fixed crypts in mice described in **e**. (n=4 for each treatment group). **g**, Quantification and classification of β-catenin^{+} clonal lesions from mice described in **e**. (n=4 for each treatment group). **h**, Representative images of *NOTUM*-ISH and β-catenin IHC on serial sections from human colonic adenoma and surrounding normal mucosal tissue. Note, NOTUM/β-catenin double-positive cells only observed in the adenoma tissue. Scale bar, 20 μm. **i**, Relative percentages of *NOTUM*-intensity within positive regions of adenoma and neighbouring mucosal tissue from human FAP-patient samples. **j**, Schematic depicting the proposed model of Notum-mediated Wnt inhibition of wild-type ISCs (green) by *Apc*-mutant cells (brown). Curved arrows indicate activation and blunt-ended arrows inhibition. Arrow thickness resembles strength of activity. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. $P<0.05$ is considered significant.

Extended Data Figure 6. Notum drives the elimination of wild-type cells from the crypt independent of apoptosis.

a, Quantification and representative confocal image of cleaved caspase-3^{+} cells (CC3) within clonal crypts and the surrounding non-mutant epithelium in *Notum*^{WT} and *Notum*^{cKO} mice 14 d.p.i . n=4 of each genotype. Crypts adjacent (Adj.) to or remote from (Rem.) *Apc*-mutant (clone) crypts were scored as non-mutant epithelia. Red arrows indicate CC3^{+} cells (green) in areas of *Apc*-mutant clones (purple border) and surrounding epithelia. Scale bar, 50 μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. *P*<0.05 is considered significant.

Extended Data Figure 7. Cells that escape *Notum* deletion upregulate *Wif1*, but not *Dkk3*.

a, Quantification of the relative percentage of *Notum*$^{+}$ adenomas (as detected via *Notum*-ISH) in *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{+/+}$ (+/+) and *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ (fl/fl) mice sampled 85 days of age. Mice were given 2 mg tamoxifen induction at 6 and 8 weeks of age. n=11 *Notum*$^{+/+}$, n=8 *Notum*$^{fl/fl}$ mice. **b**, Representative RFP-IHC of *VilCre*^{ER}*;Apc*$^{Min/+}$;*tdTom*$^{+}$ tumour tissue to show recombination efficiency at 85 days of age. Mice were given 2 mg tamoxifen induction at 6 and 8 weeks of age. Boxed areas show close-ups of three separate tumours (1-3) from a single *VilCre*^{ER}*;Apc*$^{Min/+}$;*tdTom*$^{+}$ animal. Scale bar, 10 mm. **c**, Serial sections of intestinal tumour tissue stained via ISH for *Notum*, *Wif*1 and *Dkk3* from three separate *VilCre*^{ER}*;Apc*$^{Min/+}$;*Notum*$^{fl/fl}$ mice described in **a**. Note, *Wif1* is upregulated in *Notum*-negative epithelial cells (boxed area). Scale bar, 50 μm.

Extended Data Figure 8. *Notum* is expressed by ligand-independent and not ligand-dependent tumours.

a, Relative percentages of clonal crypt classification (clonal crypt phenotype) from *Lgr5Cre^{ER};Apc$^{fl/fl}$* mice treated daily with vehicle or NOTUMi (30 mg/kg) following 0.15 mg tamoxifen induction and sampled 21 days post induction. (n=4 mice of each treatment group). **b**, Quantification of β-catenin^{+} lesions from mice described in **a**. **c**, Representative examples of high, moderate and low *NOTUM* expression (as shown via ISH) within human colonic adenoma tissue. Arrows indicate single NOTUM positive cells. Scale bar, 20 μm. **d**, Representative *NOTUM* expression, as shown by fluorescent ISH (FISH) on human colonic adenoma tissue. Tubulovillous adenoma (TVA), traditional serrated adenoma (TSA). Of note, *NOTUM* expression is minimally expressed in known RSPO-1 fusion mutant adenoma tissue (TSA). Scale bar, 50 μm. **e**, Representative *Notum*-ISH and β-catenin-IHC on *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$* mice 14 days following 2 mg tamoxifen induction. Boxed areas show close-up of nuclear β-catenin^{+}/Notum^{-} epithelium (right panels). Scale bar, 50 μm. **f**, Quantification and representative images of WT organoids treated for 5 days with conditioned medium harvested from *VilCre^{ER};Rnf43$^{fl/fl}$;Znrf3$^{fl/fl}$* organoids (*R/Z$^{-/-}$* CM) ± recombinant Notum. Organoids were derived independently from n=3 mice. Scale bar, 100μm. Data are ± s.e.m; Mann–Whitney U-test; *P* values are shown in the corresponding panels. *P*<0.05 is considered significant.