Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche

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Hereditary mixed polyposis syndrome (HMPS) is characterized by the development of mixed-morphology colorectal tumors and is caused by a 40-kb genetic duplication that results in aberrant epithelial expression of the gene encoding mesenchymal bone morphogenetic protein antagonist, GREM1. Here we use HMPS tissue and a mouse model of the disease to show that epithelial GREM1 disrupts homeostatic intestinal morphogen gradients, altering cell fate that is normally determined by position along the vertical epithelial axis. This promotes the persistence and/or reacquisition of stem cell properties in cells that have exited the stem cell niche. These cells form ectopic crypts, proliferate, accumulate somatic mutations and can initiate intestinal neoplasia, indicating that the crypt base stem cell is not the sole cell of origin of colorectal cancer. Furthermore, we show that epithelial expression of GREM1 also occurs in traditional serrated adenomas, sporadic premalignant lesions with a hitherto unknown pathogenesis, and these lesions can be considered the sporadic equivalents of HMPS polyps.

The intestinal mucosa is covered by a self-renewing layer of epithelium, making it ideal for the study of tissue-specific stem cells and cell fate determination. Lineage-tracing experiments have helped identify genes selectively expressed by intestinal stem cells. One of these marker genes, the Wnt target, leucine-rich-repeat–containing G protein–coupled receptor 5 (Lgr5), is expressed in crypt base columnar cells (CBCs) within the crypt base stem cell niche, which also comprises surrounding Paneth cells and intestinal subepithelial myofibroblasts1. In homeostasis, cell fate determination is coupled to position along the crypt–villus (vertical) axis of the epithelium. Stem cell progeny exit the niche, initially as proliferating transit-amplifying cells before progressively differentiating into post-mitotic specialized cells. This is controlled by strict gradients of interacting morphogens—soluble molecules produced by a restricted region of a tissue that form an activity gradient away from source. The phenotypic response of a cell is determined by its position within this concentration gradient2. Wnt and bone morphogenetic protein (BMP) pathways form polarized expression gradients along the epithelial vertical axis. Stem cell division and transit-amplifying cell proliferation are driven by high Wnt and low BMP levels in the lower half of the crypt, whereas daughter cell differentiation and apoptosis are controlled by low Wnt and high BMP at the luminal surface3. These gradients are maintained partly by diffusion of ligands but also by the restricted paracrine secretion of ligand-sequestering BMP antagonists, such as Gremlins, Gremlin2 and Noggin, that are exclusively derived from subcrypt myofibroblasts and act locally within the crypt base stem cell niche. These antagonists are thought to prevent BMP activity within the niche, promoting intestinal stem cell function4.

Disregulation of the homeostatic Wnt-BMP balance can promote intestinal tumorigenesis. The conventional adenoma–carcinoma sequence is commonly initiated by activation of Wnt signaling in the epithelium through adenomatous polyposis coli (APC) or β-catenin (CTNNB1) mutations5. However, disrupted BMP signaling can also predispose to intestinal polyps and cancer6. Human juvenile polyposis syndrome (JPS) results from inactivating germline bone morphogenetic protein receptor 1a (BMPRIA) or Mothers against decapentaplegic homolog 4 (SMAD4) mutations, and epithelial expression of Nog under the control of villin (Vil1) or fatty-acid–binding protein (Fabp1) regulatory elements causes a JPS-like phenotype in mice7,8. Recently, we demonstrated that human HMPS is caused by a 40-kb genetic duplication that results in aberrant epithelial expression of GREM1, disrupting homeostatic intestinal morphogen gradients, altering cell fate that is normally determined by position along the vertical epithelial axis.
duplication upstream of the BMP antagonist GREM1, which results in ectopic epithelial gene expression and resultant BMP signaling antagonism throughout the vertical axis of the intestine (Supplementary Fig. 1c–e). HMPS is an autosomal dominant condition, and untreated individuals develop colorectal cancer at a median age of 47 (ref. 10). HMPS is named for the distinctive morphology of the polyps, with individuals developing colorectal cancer at a median age of 47 (ref. 10).

Although it has recently been shown that scarce, post-mitotic tuft cells can persist outside the stem cell niche, the majority of differentiated cells (enterocytes, colonocytes and goblet cells) are shed into the lumen within 5 d. As a consequence of this rapid cell turnover, the perpetual stem cell at the crypt base has been considered the cell of origin of colorectal cancer (CRC) (Fig. 1a). Here, we use a mouse model of HMPS to show that disruption of homeostatic BMP gradients by aberrant epithelial expression of Grem1 alters cell fate determination, allowing cells outside the crypt base stem cell niche to act as tumor progenitors. Furthermore, we demonstrate that this is the pathogenic mechanism underpinning the development of human HMPS polyps and some sporadic intestinal tumors.

RESULTS

HMPS polyps are characterized by ectopic crypt foci formation

All crypts in individuals with HMPS have epithelial GREM1 expression, yet the polyps are discrete, often containing mixed dysplastic and nondysplastic areas. Histopathological review of the polyps revealed ectopic crypt foci (ECFs) that developed orthogonally to the crypt axis and contained actively proliferating cells (Fig. 1a–c and Supplementary Fig. 1b). Within some polyps, we identified dysplastic cells emerging from ECFs rather than from the crypt base (Fig. 1b) and hypothesized that dysplasia resulted from somatic mutations within the ECFs.

![Figure 1](image-url)

Figure 1 Human HMPS polyps. (a) H&E staining of an HMPS polyp showing mixed adenomatous, serrated and dilated cyst morphology and close up of ectopic crypts growing orthogonally to crypt axis. (b) Dysplastic cells (black arrowhead) emerging from an ectopic crypt rather than from the crypt base. (c) Top, immunostaining of HMPS polyps showing patchy loss of phosphorylated SMAD1, SMAD5 and SMAD8, Ki-67 stain in proliferating crypt foci cells and ectopic lysozyme stain in dysplastic crypts. Bottom, SOX9 and EPHB2 immunostaining is increased whereas staining for the differentiation marker CK20, is lost in the ectopic crypt foci of HMPS polyps (n = 23 polyps for all stains).

(d) Candidate gene (epi)genetic mutation spectra in HMPS polyps. (e) Laser-capture isolation of individual crypts across HMPS lesions. Spatial distinction of mutant clones allowed inference of mutation timing (see also Supplementary Fig. 2). Scale bars are 100 μm unless otherwise stated.

Sanger sequencing of candidate genes in 23 polyps from 14 patients with HMPS revealed a high frequency of CRC driver mutations. We observed mutually exclusive KRAS or BRAF mutations in 100% of lesions and APC, predominantly p.Arg1450X, mutations in 48%. The CpG island methylator phenotype (CIMP) was present in 53% of lesions tested (Fig. 1d and Supplementary Fig. 2a). In contrast, we found a very low frequency of known driver mutations in a cohort of JPS polyps with germline BMPRIA mutations (Supplementary Fig. 2b). Clonal ordering following microdissection of individual HMPS crypts revealed clonal KRAS or BRAF mutations detected throughout entire polyps, including ECFs and the different morphological crypt subtypes (Supplementary Fig. 2d,e). In contrast, APC mutations were often spatially restricted to dysplastic areas, indicating that Wnt dysregulation was a subsequent event (Fig. 1e and Supplementary Fig. 2a,c).

A mouse model of HMPS

In order to understand more about the pathogenesis of HMPS, we generated Vil1-Grem1 mice expressing mouse Grem1 cDNA under the control of the intestinal epithelium–specific Vil1 promoter. Epithelial expression of Grem1 was confirmed by in situ hybridization (Fig. 2c) and quantitative RT-PCR (qRT-PCR), with highest levels in the proximal small bowel (Supplementary Fig. 3d). We assessed BMP signaling using BMP ligand and target gene expression (Supplementary Fig. 3e) and immunohistochemistry for phosphorylated Smad1, Smad5 and Smad8. There was loss of the normal p-Smad1, p-Smad5 and p-Smad8 staining pattern throughout the vertical axis of the intestines of Vil1-Grem1 mice (Fig. 2d and Supplementary Fig. 4b).

Transgenic animals’ small intestines were 28% longer than those of their wild-type littermates (n = 10, P < 0.001, t-test), partly owing to an increase in the size and cell count of the villi (P < 0.001, t-test) (Fig. 2a and Supplementary Fig. 4a). Although there was no change in the proportion of Ki-67–positive proliferating cells in the crypts,
there were significantly more proliferating cells on the villi of Vil1-Grem1 animals compared to wild-type littermates ($P = 0.038$, t-test, Fig. 2a,d). Analysis of epithelial cell lineages showed a decrease in the number of goblet cells in both small intestinal ($P = 0.01$, t-test) and colonic ($P = 0.002$, t-test) crypts. The presence of lysozyme-positive Paneth cells on the villus ($P = 0.038$, t-test) of Vil1-Grem1 mice was notable as this is a cell type normally restricted to the small intestinal crypt base (Fig. 2a,d). In 3-month-old Vil1-Grem1 animals, Grem1-expressing ectopic crypts were seen developing orthogonally to the vertical axis of the widened and flattened small bowel villi (Fig. 2b,c). Ectopic crypts budded off to become actively proliferating intravillus lesions, which subsequently developed dysplastic features with concomitant loss of p16INK4A expression (Fig. 2e). By 7 months, these lesions had progressed to a pan-intestinal polyposis (Supplementary Fig. 4c), with a median of 183 polyps per mouse. Small intestinal lesions had a mixed serrated, adenomatous and cystic phenotype characteristic of the lesions seen in HMPs (Fig. 2b).

We observed epithelial Grem1 and membranous β-catenin expression in unaffected intestine and small polyps in the Vil1-Grem1 mice but some larger polyps with more advanced dysplasia exhibited marked downregulation of epithelial Grem1 expression, which correlated with foci of cytoplasmic and nuclear β-catenin staining. Sanger sequencing demonstrated activating Ctnnb1 mutations in some of these lesions (Fig. 2f and Supplementary Fig. 4d).

Although the total colonic length was unchanged in Vil1-Grem1 mice, the proximal colonic folds were exaggerated in comparison with wild-type littermates. In Vil1-Grem1 mice hyperplastic-appearing colonic crypts had an increased cell count ($P = 0.03$, t-test, Fig. 2a). Crypt crowding meant that ECFs could not be easily distinguished in the colon, but colonic dysplasia originated at the luminal surface and progressed to form lesions that contained all three morphological crypt phenotypes (Fig. 2b).

These data indicate that aberrant epithelial Grem1 expression results in a progressive intestinal polyposis with small intestinal and colonic
polyps containing the three characteristic morphologies seen in human HMPS lesions. Throughout the bowel, early lesions can be seen developing outside the crypt basal stem cell niche: on the luminal surface in the colon and within ECFs that bud into the villus of the small intestine. Downregulation of epithelial expression in some advanced lesions indicates that epithelial Grem1 is no longer required once epithelial somatic mutation events have occurred.

**Vil1-Grem1 mice have enlargement of the progenitor cell pool**

As the villus ECFs appeared to be the origin of small intestinal dysplasia in the Vil1-Grem1 mice, we looked for increased expression of stem cell markers on the villi of these animals. We crossed Vil1-Grem1 mice with Lgr5-EGFP-IRES-CreERT2 reporter mice but were unable to detect discrete Lgr5-EGFP–positive cells outside of the crypt base stem cell niche, with none seen in the villus ECFs (Fig. 3a). To confirm this, we mechanically separated crypts and villi from Vil1-Grem1 and age-matched wild-type mice and used qRT-PCR to detect stem cell markers aberrantly expressed in the Vil1-Grem1 mouse villus compartment. Of the stem cell markers tested, Sox9 showed the highest expression in villus cells (Fig. 3b), and immunostaining for Sox9 confirmed ectopic crypt-specific expression in both human HMPS and Vil1-Grem1 mouse tissue (Figs. 1c and 2d).

Next, we examined the global mRNA expression profiles of Vil1-Grem1 versus wild-type mouse crypt and villus compartments using gene set enrichment analysis (GSEA). GSEA confirmed no significant enrichment of Lgr5-based intestinal stem cell profiles on the villi of Vil1-Grem1 transgenic mice. In contrast, there was enrichment of villus gene programs characterizing proliferating early transit-amplifying cells (normalized enrichment score (NES) 7.35, P < 0.001, Kolmogorov–Smirnov test) with concomitant reduced levels of genes normally expressed in differentiating cells (NES –6.46; P < 0.001). Furthermore, we also observed reduced expression of villus genes regulating apoptosis (NES –2.9; P = 0.008), cellular senescence (NES –2.36; P = 0.026) and autophagy (NES –3.89; P < 0.001) (Fig. 3c), homeostatic processes that have all been shown to have tumor suppressor roles in early lesions.

A decreasing gradient of ephrin type-B receptor 2 (EphB2) expression from the crypt base along the vertical axis of the intestine has been used to distinguish intestinal stem (EphB2high), progenitor (EphB2medium) and differentiating (EphB2low) cell populations in the mouse and human intestine. Consistent with this and our GSEA findings, EphB2 protein was aberrantly expressed in the ECFs of both Vil1-Grem1 transgenic mouse and human HMPS polyps (Figs. 1c and 2d). Protein expression of cytokertatin 20 (CK20), a marker of differentiated cells, was reduced or absent in the ECFs of both Vil1-Grem1 mouse and human HMPS polyps (Figs. 1c and 2d).

Taken together, these data indicate that epithelial Grem1 expression disrupts the coupling of cell fate to position along the vertical axis of the intestine. Although cell fate–position uncoupling does not generate an ectopic Lgr5-positive stem cell population, it does cause expansion of an Lgr5-negative proliferating progenitor cell population on to the villi. Concomitant downregulation of differentiation, apoptosis, senescence and autophagy gene programs means there is a marked expansion of a progenitor cell pool outside of the intestinal stem cell niche that can be defined immunohistochemically as Sox9+ EphB2+CK20- (Figs. 1c and 2d).

**Epithelial Grem1 promotes persistence of somatically mutant villus cells**

In order to test the clonogenic, tumor-forming potential of villus cells in our Vil1-Grem1 mouse model, we used the established in vitro enteroid technique that uses media supplemented by the niche-derived morphogens, epidermal growth factor (E), Noggin (N) and R-spondin (S) (ENS medium). Normal-appearing crypts
Fig. 4 In vitro villus cell clonogenicity. (a) Heatmap showing in vitro culture of different tissue compartments taken from different genotype mice in varying medium conditions. Blue boxes indicate successful culture of structures with the described morphology. Red boxes indicate failure to establish tissue culture. ENS medium was considered standard conditions. Illustrated results are representative of three separate experiments. Cell culture images show development of spheroid structures from Vil1-Grem1; ApcMin/+ villi with dissociation of villi from age-matched wild-type littermates in order to assess whether aberrant epithelial Grem1 expression influenced the tumor-forming capacity of villus cells. In the presence of epidermal growth factor, Noggin, R-spondin and recombinant Wnt3a (W) (ENSW medium), clonogenic cystic spheroids did develop from Vil1-Grem1 mouse villi, but these were rare events (<0.1% of villi). We reasoned that the inefficiency of this transformation resulted from the short half-life of Wnt3a in the medium. To counter this, we generated Vil1-Grem1; ApcMin/+ mice to activate the endogenous Wnt pathway. In extracted villi, germline ApcMin/+ mutation did induce an increase in villus Wnt target gene expression (Fig. 4b). However, epithelial Grem1 reduced the expression of these Wnt targets in an Apc-mutant background while increasing the expression of the progenitor markers Sox9 and EphB2 (Fig. 4b). These results were consistent with our GSEA findings.

We then repeated villus culture taking care to sample from regions without microscopically visible polyps. Villi from wild-type and age-matched ApcMin/+ mice did not survive in culture. By contrast, villi from Vil1-Grem1; ApcMin/+ animals rapidly formed clonogenic spheroids that could be successfully propagated in long-term culture and were unaffected by the addition of competing BMP ligands and to the media. These results were consistent with our GSEA findings.
Spheroids formed from mice as young as 23 d old, but transformation efficiency increased with the age of the mouse, and histological analysis showed that this coincided with the emergence of villus ECFs (Fig. 4e).

Taken together, these results suggest that modest Wnt activation induced by a single Apc mutation alone is insufficient for the clonogenic growth of villus spheroids in nondysplastic ApcMin/+ vili. However, epithelial Grem1-induced disruption of cell fate allows the persistence of progenitor cells on the villus, and if Wnt-activating somatic mutations are present within this accumulating, expanded progenitor population, these cells are capable of generating BMP antagonist–independent spheroid growth in the cell culture environment.

Grem1 and activated Wnt signaling act in a synergistic fashion

Crossing Vill-Grem1 mice with ApcMin/+ mice caused a profound exacerbation of intestinal tumorigenesis, with tumor burden in 2-month-old double-transgenic animals greater than that seen in animals with the individual parental mutations (Fig. 5a). Vill-Grem1; ApcMin/+ mice had to be killed at mean 57 d as opposed to 200 d for ApcMin/+ mice and >250 d for Vill-Grem1 animals. Notably, morphological elements of the polyps from both parental strains could be seen in individual lesions in double-transgenic animals. In the colon, superficial aberrant crypt foci progressed to lesions with luminal surface, not crypt base dysplasia. In the small bowel, dysplasia arose from intravillus ectopic crypts contained within nondysplastic serrated epithelium (Fig. 5b and Supplementary Fig. 5a). Laser dissection of these different morphological elements demonstrated somatic loss of the wild-type Apc allele exclusively in the dysplastic crypts within the serrated villi (Fig. 5b), indicating that somatic Apc inactivation provides a selective advantage and occurs rapidly in actively proliferating villus cells.

Grem1 knockout reduces Wnt-driven tumor progression

Next, we induced widespread, multicompartmental Grem1 knockout in 6-week-old mice. To do this we used a tamoxifen-inducible, Cre-mediated recombination system driven by the chicken β-actin promoter and cytomegalovirus early enhancer, crossed with a homozygous Grem1-floxed mouse (Cagg-CreERT2; Grem1fl/fl mice).24 We confirmed successful Grem1 knockout by qRT-PCR and in situ hybridization, but Cagg-CreERT2; Grem1fl/fl animals had no consistent change in the expression of BMP constituents, Wnt targets or stem cell markers and developed no pathological phenotype, indicating possible functional redundancy or buffering of BMP antagonists in adult intestinal homeostasis (Supplementary Fig. 6). As we and others have seen epithelial and/or stromal upregulation of Grem1 in some, but not all, sporadic human intestinal polyps, cancers and cell lines,25–27 (Supplementary Figs. 5d and 7a), we examined the effect of knockout of physiological Grem1 expression on Wnt-initiated tumor burden by crossing Cagg-CreERT2; Grem1fl/fl mice with ApcMin mice. Grem1 knockout caused a significant reduction of ApcMin/+ mouse polyp burden ($P_{interaction} < 0.002$ for all regions of the bowel, linear regression, Fig. 5c) and size ($P_{interaction} < 0.001$, linear regression,
Supplementary Fig. 6d), demonstrating that knockout of stromal and/or aberrant epithelial Grem1 expression ameliorates the development of conventional intestinal tumorigenesis. To investigate whether GREM1 might influence the behavior of human tumors, we analyzed associations between GREM1 expression and survival in two publicly available CRC data sets. Individuals with above-median GREM1 expression had significantly shorter disease-free survival in both data sets (AMC-AJCCII-90 set, stage 2: log-rank $P = 0.0162$, Moffit-Vanderbilt-Royal Melbourne set, stages 1–3: log-rank $P = 0.0112$) (Fig. 5d and Supplementary Fig. 5e).

Collectively, these results strongly support the notion that GREM1 and Wnt signaling act in a synergistic fashion in the initiation and progression of intestinal polyps. We propose that in both human and mouse neoplasia resulting from aberrant GREM1 expression, there is a strong selective pressure for somatic Wnt activation in actively dividing ECFs, whereas in conventional Wnt-driven tumorigenesis, stromal-derived GREM1 provides a favorable microenvironment for the persistence of somatically mutant cells.

Figure 6 Model summarizing the proposed mechanistic consequences of disrupted GREM1 morphogen gradients. Aberrant ectopic epithelial expression of GREM1 disrupts the coupling of cell fate determination to position along the crypt-villus axis and allows persistence and expansion of an Lgr5-negative progenitor cell pool (characterized by aberrant SOX9 and EPHB2 expression) that forms orthogonal ectopic crypt foci. Aberrant cell proliferation in this progenitor cell population within these ECFs predisposes toward somatic (epi)mutation events and gives rise to neoplastic transformation (inset boxes). In vitro, the persistence of somatically mutated progenitor cells in dissected villi gives rise to clonogenic tumor spheroid growth from cells that have exited the crypt basal stem cell niche. Colored bars represent morphogen and gene expression gradients in the normal and pathological states. Blue squares represent physiological Grem1 expression from pericryptal myofibroblasts. CBC stem cells are colored red.

Epithelial GREM1 expression in sporadic traditional serrated adenomas

On the basis of gene expression and somatic mutation profiles, a recent study divided sporadic colorectal cancer into three main molecularly distinct subtypes: CCS1, enriched for tumors with chromosomal instability; CCS2, enriched for tumors with microsatellite instability; and a third group, CCS3. This group was more heterogeneous, appeared to arise from serrated adenoma precursors and gave rise to an aggressive subset of cancers with poor prognosis. To see whether whole-tumor GREM1 expression correlated with this classification, we used The Cancer Genome Atlas RNA-seq data to match sporadic tumor samples to the published CCS1, CCS2 and CCS3 subtypes and found a highly significant correlation between high GREM1 expression and CCS3-subtype tumors ($P < 0.0001$, analysis of variance (ANOVA)) (Fig. 5d).

Traditional serrated adenomas (TSAs) are distal colonic polyps with hitherto unknown pathogenesis that make up about 2% of the lesions removed at colonoscopy. The characteristic histological feature...
of these sporadic lesions is the development of ectopic crypt foci\(^2\). We collected and dissected individual crypts from four fresh TSA specimens. Individual crypt analysis of BMP antagonist expression revealed a mean 87-fold upregulation of epithelial GREM1 expression over surrounding normal mucosa (Fig. 5e), which was significantly greater than that seen in conventional, hyperplastic and sessile serrated adenomas (\(P < 0.01\), t-test, Supplementary Fig. 7a). We validated epithelial GREM1 expression in a set of ten paraffin-embedded TSAs using mRNA in situ hybridization. We detected clearly visible epithelial GREM1 expression in a further six of ten polyps (Fig. 5f). Immunohistochemical assessment, mutation and methylation analysis revealed a very similar molecular phenotype and somatic (epi)mutation pattern to HMPS polyps (Supplementary Fig. 7b,c). We observed CIMP in 33% of TSAs, and p16\(^{INK4A}\) promoter methylation correlated with p16\(^{INK4A}\) protein downregulation and resultant cell proliferation similar to the p16\(^{INK4A}\) and Ki-67 staining observed in Villi-Grem1 mice (Supplementary Fig. 7d). These data suggest that the ECFs that characterize sporadic TSAs also arise from disruption of homeostatic morphogen gradients and that in the majority of cases this is the consequence of aberrant epithelial GREM1 expression.

**DISCUSSION**

Here we use a mouse model of a human disease to demonstrate the pathogenetic mechanism in hereditary mixed polyposis syndrome (Fig. 6). We hypothesize that aberrant epithelial GREM1 expression disrupts intestinal morphogen gradients, altering daughter cell fate and promoting the persistence of Lgr5-negative progenitor cells in ECFs distant from the crypt base. Cell proliferation within ECFs means that progenitor cells are prone to tumor-causing somatic mutations. Human HMPS polyps progress through KRAS or BRAF mutation and frequent selection of a very restricted set of APC mutations. In the mouse model, Grem1-initiated lesions advance through p16 loss and Ctnnb1 mutation, and once somatic mutation has occurred in some advanced lesions, epithelial Grem1 expression becomes redundant. *Ex vivo* culture of transgenic mouse villi demonstrates that persistence of somatically mutated villus cells can initiate clonogenic growth. Using mouse models, we have also shown the exacerbation or amelioration of conventional, Wnt-driven neoplasia initiation by Grem1 overexpression or knockout, respectively. Lastly, we show that epithelial expression of Grem1 also occurs in sporadic TSAs, lesions similarly characterized by the development of aberrantly proliferating cells in ectopic crypt foci, and that these lesions can thus be considered the sporadic equivalents of HMPS polyps.

Multiple BMP antagonists have been described, with varying levels of intestinal expression and importance in intestinal homeostasis. There is much greater physiological expression of both GREM1 and GREM2 than of NOG in human intestinal stroma (Supplementary Fig. 5f), and the importance of GREM1 has been highlighted by its causative role in HMPS\(^9\) and the association of sporadic CRC with GREM1 common allelic variants\(^30\). The association of germline-inactivating NOG mutations with skeletal conditions such as symphalangism and tarsal-carpal coalition\(^31\) correspondingly reflects the greater significance of NOG\(^s\) role in bone development than in intestinal homeostasis. Previous investigation of the pathogenesis of juvenile polyposis led to the generation of mice expressing *Xenopus nog* under control of the VillI promoter\(^2\) and mice expressing *Xenopus nog* under control of the Fabp1 promoter\(^8\). Both models initially developed intravillus ectopic crypts before progressing to a juvenile polyposis–like phenotype. The differences between small intestinal polypl morphology in these models and in the Vill1-Grem1 mouse may reflect the *Xenopus* origin of the *nog*, the relative importance of these different antagonists in intestinal homeostasis or fundamental differences in the ligand targets or biology of these BMP antagonists, which share minimal sequence homology. Human HMPS and JPS are pathogenically and morphologically distinct\(^32\), and the profound differences in pathogenesis, phenotype and somatic pathway progression between these conditions highlights how subtle alterations in the BMP signaling cascade can have different effects on intestinal tumorigenesis.

The histogenesis of colorectal neoplasia has historically been a subject of some debate: the classical bottom-up model favors dysplasia arising from the crypt basal stem cell niche\(^3\), whereas the top-down model proposes that dysplasia originates at the luminal surface and spreads downwards\(^34\). The identification of the Lgr5-positive CBC stem cell at the very base of the crypt\(^3\) seemed to have settled the argument in favor of the bottom-up model. Here, we demonstrate that pathological disruption of morphogen gradients in animal models and human inherited and sporadic disease causes a profound change in the fate of cells situated outside of the crypt base stem and proliferative zone, with expansion of an Lgr5-negative progenitor cell population. These cells form ectopic crypt structures, proliferate, accumulate somatic mutations and are capable of initiating intestinal neoplasia, indicating that the crypt basal stem cell is not the exclusive cell of origin of all subtypes of colorectal cancer. Conceivable histogenetic origins of this progenitor population include the vertical expansion of a crypt base progenitor population containing variably competent stem cells, the generation of an ectopic niche for a migrated Lgr5-negative stem cell and dedifferentiation of post-mitotic specialized intestinal epithelial cells. Very recently, Schwitalla et al.\(^38\) have shown that activated nuclear factor-xB–induced mucosal inflammation in combination with constitutive epithelial Wnt signaling can also promote the initiation of neoplasia from cells situated outside the crypt base stem cell niche. Taken together, these studies highlight the importance of the intestinal microenvironment in maintaining stem cell homeostatic control and cell-fate determination. This work challenges the concept of a strict unidirectional tissue organizational hierarchy in the intestine and demonstrates that a top-down model of tumor histogenesis may fit some subtypes of inherited, sporadic and inflammation-associated colorectal cancers.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Gene expression data have been deposited in the NCBI Gene Expression Omnibus with accession number GSE62307.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

S.J.L. and I.T. conceived and designed the project. Experiments were conducted by H.D., S.I., H.R., T.B., C.B., E.J., A.L., P.R.-C. and S.J.L. In situ hybridization was completed by H.D., R.J., R.P. and A.S. Bioinformatic analysis carried out by S.I., M.B., L.F.-M. and E.C.G. Pathology support, tissue provision and intellectual
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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Generation and genotyping of Vil1-Grem1 mice. Grem1 cDNA was amplified from normal mouse intestinal mesenchyme using the following primers F 5′-GAGCTGACCAATGAGAGAC-3′ and R 5′-GACGTCTGAGCAGAAGC-3′, adding an AatII restriction enzyme site at each end. The excised cDNA fragment was then cloned into the Villin-MES-SV40polyA plasmid (kind gift from S. Robine) downstream of the 9-Kb Villin promoter (active after embryonic day E9). This plasmid was linearized with Kpn1 digestion. Transgenic mice were derived by pronuclear injection in BL6/129 mice using standard methods by the Transgenic Facility, WTCHG, Oxford University. To identify successful transmission, tail snips were taken from mice at weaning age and amplified by PCR using the following primers: F 5′-GAGCTGAGGAGTCCAGG-3′ and R 5′-GACGTCTGAGCAGAAGC-3′ and R 5′-AC CTAGTTCGGCCATCTCC-3′. Successful transmission was confirmed by Southern blot using standard methods. In brief, the probes were amplified using the following primers F 5′-AGTGGGAGGCTGAGGCTA-3′ and R 5′-CAACGCTCCCAAGTGTATG-3′. The probes were then radioactively labeled by random priming with [32P]dCTP using the random primed labeling kit (Roche) as previously described.17 20 μg of genomic DNA was digested to completion with SphI and MscI restriction enzymes. The digested DNA was electrophoresed on 0.8% gels in 1× TBE overnight. After denaturation, the gels were blotted onto Hybond N+ membrane (GE Healthcare) overnight, UV crosslinked (Stratalinker, Stratagene) and hybridized with the radiolabeled probes. Hybridization and washing of Southern blots was performed using standard methods and detected a 2,079-bp region of the inserted transgene in experimental and control mice, a total of three mice per group were used. Similarly, 100 crypts of the colon were counted, giving us enough power to run a t-test. Data are represented as total number of cells per crypt/villus. Excel was used to calculate means and standard error. To determine the proliferative index in experimental and control mice, a total of three mice per group were used. Small intestinal and colon sections were immunohistochemically labeled with Ki-67–specific antibody. Microscopic images at ×20 magnification were taken and number of Ki-67+ positive cells per total epithelial cells in randomly selected 50 crypts (colon or small intestine) and 50 villi (small intestine) were counted. Data are represented as a percentage of Ki-67+ cells in each compartment. The percentage of goblet cells in epithelial cells of the villus (small intestine) and crypt (small intestine/colon) was quantified by counting Alcian blue–positive cells in at least 50 crypts of the colon/small intestine and 50 villi from small intestine in three mice of each genotype. Similarly, quantification of Paneth cells was determined by counting lysozyme-positive cells over total epithelial cells in three Vil1-Grem1 and three wild-type mice. To assess apoptosis, cleaved Caspase 3–positive cells over total number of epithelial cells per crypt (50 crypts in total) in small intestine/colon, and per villus (50 villi in total) in small intestine were counted. No randomization or blinding of mouse genotypes was used. All statistical analyses were done in Excel.

Immunochemistry. Formalin-fixed, paraffin-embedded tissue sections (4 μm) were de-waxed in xylene and rehydrated through graded alcohols to water. Endogenous peroxidase was blocked using 1.6% H2O2 for 20 min. For antigen retrieval, sections were pressure cooked in 10 mmol/L citrate buffer (pH 6.0) for 5 min. Sections were blocked with 10% serum for 30 min. Slides were incubated with primary antibody for 2 h. Antibodies to the following proteins have been used in this study: alkaline phosphatase (Abcam, ab68343, 1:50), β-catenin (BD, 610154, 1:50), Caspase 3 (R & D, AF835, 1:800), Cdkn2a/ p16INK4a (mouse) (Cell Signaling, 9511L, 1:50), Cagg-CreER2 and Grem1βGal (ref. 39) mice have previously been reported.

Sections were then incubated in ABC (Vector labs) for 30 min. DAB solution was applied for 2–5 min and development of the color reaction was monitored microscopically. Slides were counterstained with hematoxylin, dehydrated, cleared and then mounted.

Alcian-blue stain for goblet cells. Sections were dewaxed in xylene for 5 min and then rehydrated through graded ethanol (100%, 90%, 70%) for 5 min each followed by 2 min in tap H2O. Slides were then stained in alcian-blue solution (Sigma) for 30 min, washed in running tap H2O for 2 min, and rinsed in dH2O. Slides were then stained in nuclear fast red solution (Sigma) for 5 min and washed in running tap H2O for 1 min. Slides were then dehydrated through degraded alcohols for 2–5 min each, before mounting a coverslip with DPX.

Immunohistochemical quantification. For mouse phenotype quantification analyses, we used no fewer than three animals per group (control and experimental, at age 10 months). To assess overall change in epithelial cell numbers between Vil1-Grem1 mice compared to age-matched, wild-type counterparts, we microscopically captured images of hematoxylin-eosin–stained 4-μm sections of the small intestine and colon at ×10 magnification and then randomly selected crypts and villi, counting individual cells within these compartments. An average of 50 individual villi within the small intestine and 100 crypts were counted. Similarly, 100 crypts of the colon were counted, giving us enough power to run a t-test. Data are represented as total number of cells per crypt/villus. Excel was used to calculate means and standard error. To determine the proliferative index in experimental and control mice, a total of three mice per group were used. Small intestinal and colon sections were immunohistochemically labeled with Ki-67–specific antibody. Microscopic images at ×20 magnification were taken and number of Ki-67+ positive cells per total epithelial cells in randomly selected 50 crypts (colon or small intestine) and 50 villi (small intestine) were counted. Data are represented as a percentage of Ki-67+ cells in each compartment. The percentage of goblet cells in epithelial cells of the villus (small intestine) and crypt (small intestine/colon) was quantified by counting Alcian blue–positive cells in at least 50 crypts of the colon/small intestine and 50 villi from small intestine in three mice of each genotype. Similarly, quantification of Paneth cells was determined by counting lysozyme-positive cells over total epithelial cells in three Vil1-Grem1 and three wild-type mice. To assess apoptosis, cleaved Caspase 3–positive cells over total number of epithelial cells per crypt (50 crypts in total) in small intestine/colon, and per villus (50 villi in total) in small intestine were counted. No randomization or blinding of mouse genotypes was used. All statistical analyses were done in Excel.

Individual crypt and villus isolation, RNA extraction and qRT-PCR. For both mouse and human individual crypt/villus isolation, biopsies were washed with PBS and incubated in 5 ml dissociation media (30 mM EDTA in DMEM without Ca2+ and Mg2+, 0.5 mM DTT, 2% RNAlater (Life Technologies) for 10 min at room temperature. The digested tissue was then transferred to PBS and shaken vigorously for 30 s to release individual crypts and villi. Individual structures were selected using a drawn-out glass pipette under a dissection microscope and transferred to RTL buffer ready for subsequent RNA extraction with the RNAeasy microkit (Qiagen) according to manufacturer’s instructions. RNAs were treated with DNase I to degrade residual DNA. When required, complementary DNA was reverse transcribed in vitro using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). When using cDNA generated from individual crypts or villi, preamplification of these cDNAs was necessary before qRT-PCR. The TaqMan PreAmp (Applied Biosystems) kit was used following manufacturer’s instructions. Absolute quantification qRT-PCR was performed on the ABI 7900HT cycler (Applied Biosystems) with GAPDH/Gapdh serving as an endogenous control. A list of TaqMan Gene Expression assays (Applied Biosystems) used is available on request. The primary assumption in analyzing Real time PCR results is that the effect of a gene can be adjusted by subtracting Ct number of target gene from that of the reference gene (ΔCt). The ΔCt for experimental and control can therefore be subject to s test, which will yield the estimation of ΔΔCt. In all cases the data met the normal distribution assumption of the t-test.

In situ hybridization. 4-μm sections were prepared using DEPC (Sigma)–treated H2O. In situ hybridization was carried out using the GREM1 (312831), PPBP...
Mouse Apc loss-of-heterozygosity. Apc allele status was assessed by a PCR assay described previously using primers F 5′-TCTGCTTTGAGAAGACA GAAACT-3′ and R 5′-TAGATCTCCTCAGTTTGTCCTAT-3′ (ref. 41). PCR products were electrophoresed on 2.5% agarose gels. Briefly, the amplification of the ApcMin allele resulted in a 155-bp PCR product with one HindIII site, whereas the 155-bp product for the Apc+ allele contained two HindIII sites. HindIII digestion of PCR-amplified DNA from ApcMin/+ heterozygous tissue generates 123-bp and 144-bp products. PCR products from tissue with LOH were exposed to UV light for 30 min before mounting 8-µm sections. These slides were baked at 37 °C for 30 min and then dewaxed for 5 min and rehydrated through graded alcohols to water for 3–5 min each. The slides were then briefly dipped in methyl green, washed in water and dried at 37 °C for 1 h. Laser capture microdissection was performed with the laser capture PALM system (Zeiss). DNA was extracted using the PicoPure DNA extraction kit (Arcturus).

Sequencing. Sequencing of gDNA was carried out using the 2x Big Dye Terminator v3.1 reagent (Applied Biosystems). Unincorporated dye terminators were removed with the DyeEx 2.0 Spin kit (Qiagen) and the purified products were run on the ABI 3730 DNA analyzer (Applied Biosystems). Sanger sequencing primers of candidate genes are as follows: Ctnn1 5′-E2FTCTACGAGATTTTCCGTCG CAC, Ctnn1 3R TAGTCTCACAACAAATGTC, Kras/12/13 GTGCACATGTCTCCTATATGTCC, Kras/12/13 GAATGGCTTGGTCAAGGAT, BRAF 5′ TCATAAGTCTGGGCTGGTATAG, BRAF 5′ TCTGGGCGTTGTTATATCTCC, TP53 3R TCTGGTCAAGGAT, TP53 3F TCTGGTCAAGGAT, TP53 5R AACCAGGCTCTGTCGGTCTC, TP53 6F GCCGGCTTGGTTATAG, TP53 6R TAATAGTCCTTGCTGGTATAG, TP53 7F CTTGGGCCCTGTGTTAT and TP53 7R CTTCATAGTCCTTGCTGGTAT.

Culture of mouse intestinal crypts. Mouse intestinal crypts were isolated, resuspended in Matrigel (BD Biosciences) and plated out in 24-well plates. The basal culture medium (advanced Dulbecco’s modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1× N2, 1× B27 (all from Invitrogen), and 1 mmol/L N-acetylcysteine (Sigma)) was over-laid containing the following growth factors; Epidermal Growth Factor at 50 ng/ml (Life Technologies), Noggin at 100 ng/ml (PeproTech) and R-spondin1 at 500 ng/ml (R and D) (ENS media). The medium was changed every 2 d.

Culture of intestinal adenomas. From symptomatic ApcMin/+ animals the intestines were opened longitudinally and adenomas were scraped with a coverslip. The first scrap of material was discarded and the subsequent scrapes were collected into sterile PBS. The material was gently washed with PBS and then centrifuged at 100g for 3 min. The material was then plated out in 24-well plates in Matrigel (BD Biosciences). The wells were overlaid with Epidermal Growth Factor at 50 ng/ml (Life Technologies), R-spondin1 at 500 ng/ml (R and D) and Gremlin1 at 100 ng/ml (R and D) (EGS media). After spheroids had formed (4 d of culture) Gremlin1 was subsequently withdrawn from the media and to demonstrate the necessity of BMP signaling, Vill-Grem1 crypt enteroids were grown in media lacking Noggin (ES media) together with a range of BMP ligand 2, 4 and 7 concentrations (rBMP2, rBMP4 and rBMP7 (R&D Systems) 0–1,000 ng/ml of each of the three BMP ligands). Cells cultured in media containing rBmp concentrations of 50–1,000 ng/ml of each ligand were not viable (enterospheres were formed and were still present on day 2 but completely disaggregated by day 5).

Culture of intestinal villi. Vill-Grem1 animal intestines were opened longitudinally and the villi were scraped off with a coverslip. The first scrap of material was discarded and the subsequent scrapes were collected into sterile PBS. The material was gently washed with PBS and then centrifuged at 100g for 3 min. The villi were then plated out in 24-well plates in Matrigel (BD Biosciences) and to demonstrate the necessity of BMP signaling, Vill-Grem1 crypt enteroids were grown in media lacking Noggin (ES media) together with a range of BMP ligand 2, 4 and 7 concentrations (rBMP2, rBMP4 and rBMP7 (R&D Systems) 0–1,000 ng/ml of each of the three BMP ligands). Cells cultured in media containing rBmp concentrations of 50–1,000 ng/ml of each ligand were not viable (enterospheres were formed and were still present on day 2 but completely disaggregated by day 5).
ligands, showing the overexpression of Grem1 is no longer required for the growth and maintenance of villus spheroids.

**Passaging and embedding of spheroids.** After the intestinal villi had been cultured for approximately a week and had grown into spheroid structures, they were passaged by adding cold media to melt the Matrigel and subsequently replated in fresh Matrigel. To collect material for embedding, the Matrigel was melted by adding cold media; then multiple wells were combined. The cells were fixed with 500 µl PFA 30 min at room temperature, centrifuged at 5,000 r.p.m. and resuspended in 150 µl 2% agarose (in PBS). The paraffin-embedded cell pellet was then processed using standard protocols.

**DNA extraction.** To extract DNA from crypt, villi and spheroids the QiaAmp Micro kit (Qiagen) was used following manufacturer’s instructions.

**Gene expression arrays.** For GEP study, we used n = 6 controls and n = 6 experimental groups to have a good estimate of the mean expression as well as to give us sufficient power for the t-test. Raw data from Illumina gene expression arrays (MouseWG-6_V2_0_R0_11278593_A chips) was processed after removing one outlier sample from initial quality control (detection score of < 0.95 of the background intensity for majority of probes) using the VSN (variance-stabilization and normalization) algorithm (Supplementary Table 1). We applied a filter by taking a detection score of > 0.95 of the background intensity distribution for all samples to consider a probe detectable, resulting in a total of 24,854 detectable probes. Differentially expressed genes between experimental (Vill-Grem1 small intestinal crypts (n = 6 mice)/villi (n = 5 mice)) and normal (wild-type small intestinal crypt (n = 6 mice)/villi (n = 6 mice)) were identified using Student’s t-test by running “test2” command in MATLAB (NCBI Gene Expression Omnibus with accession number GSE62307).

Gene set enrichment analysis (GSEA) was performed using Kolmogorov-Smirnov statistics and gene shuffling permutations as described16. Genes were ranked by computing their differential expression in the experimental versus normal samples by the Student’s t-test method. If multiple probes were present for a gene, probe with the highest absolute differential expression between experimental and normal was selected. We used gene shuffling with 1,000 permutations to compute the P value for the enrichment score. A list of gene signatures14–19 used in the enrichment analysis is given in Supplementary Table 3a,b. If the signature was from human data set, we mapped these human genes to their mouse orthologs using the sequence-based method available from MGI (http://www.informatics.jax.org/).

**Human colorectal cancer patients.** Two publically available patient cohorts were downloaded from the NCBI Gene Expression Omnibus (GEO) using the R BioConductor package GEOquery. The first set comprises 90 patients with stage 3 CRC treated in the Academic Medical Center in Amsterdam, defined by the GEO accession number GSE33114 (AMC-AJCCII-90 set)25. The second set contains 345 patients with colorectal cancer (CRC), defined by GEO accession numbers GSE14333 and GSE17538 (Moffit-Vanderbilt-Royal Melbourne set)26. Both sets are explained in detail in ref. 28.

Kaplan-Meier survival curves were generated using the R package survival. The median expression value of GREM1 was used to segregate patients into low- or high-GREM1 expressors. P value was calculated using the log-rank test as informative covariates for Cox proportional hazards (such as stage) were not present in all data sets and because the test is robust to the high degree of right-censored data present.

RNA-seq data from patients with sporadic colorectal cancer was downloaded from The Cancer Genome Atlas (TCGA) Data portal (http://cancergenome.nih.gov/) and normalized using Voom in Limma package. Tumor samples were matched to the published CRC subtypes CC51, CC52 and CC53, as described previously28. If for any given patient multiple samples were profiled, we randomly selected one sample for further analysis. The analysis of variance (ANOVA) test was used to compare the mean expression value of GREM1 across multiple patients. We assessed whether GREM1 expression levels in the TCGA cohort correlated with any of the CC51, CC52, CC53 sub-types.

**Ethics.** Ethical approval for use of archival HMPS tissue was provided by the Southampton and South-West Hampshire Research Ethics Committee A (REC 06/Q1702/99). Ethical approval for the collection and use of endoscopic and archival TSA samples was obtained from the Oxfordshire Research Ethics Committee A (REC 10/H0604/72.) Ethical approval for tissue use in research was obtained from all patients before endoscopic or surgical procedure. All mouse experiments were completed in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986 (Animals in Science Regulation Unit, project license PPL30/2763) and the University of Oxford Clinical Animal Welfare and Ethical Review Body.

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