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Region-specific Wnt signaling responses promote gastric polyp formation in familial adenomatous polyposis patients

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Abstract

Germline APC mutation in familial adenomatous polyposis (FAP) patients promotes gastrointestinal polyposis, including the formation of frequent gastric fundic gland polyps (FGPs). In this study, we investigated how dysregulated Wnt signaling promotes FGPs and why they localize to the corpus region of the stomach. We developed a biobank of FGP and surrounding non-polyp corpus biopsies and organoids from FAP patients for comparative studies. Polyp biopsies and polyp-derived organoids exhibited enhanced Wnt target gene expression. Polyp-derived organoids with intrinsically upregulated Wnt signaling showed poor tolerance to further induction, suggesting that high Wnt restricts growth. Targeted genomic sequencing revealed that most gastric polyps did not arise via APC loss-of-heterozygosity. Studies in genetic mouse models demonstrated that heterozygous Apc loss increased epithelial cell proliferation in the corpus but not the antrum, while homozygous Apc loss was not maintained in the corpus yet induced hyperproliferation in antrum. Our findings suggest that heterozygous APC mutation in FAP patients may be sufficient to drive polyp formation in the corpus region while subsequent loss-of-heterozygosity to further enhance Wnt signaling is not tolerated. This finding contextualizes the abundant yet benign nature of gastric polyps in FAP patient corpus compared to the rare, yet adenomatous polyps in the antrum.

Key Words: Stomach, Cellular Proliferation, Corpus, Antrum, Adenomatous Polyposis Coli
Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant disease resulting from an inherited loss-of-function mutation to the tumor suppressor adenomatous polyposis coli (APC). APC mutation leads to activation of the Wnt signaling pathway, and thus FAP patients are predisposed to developing disease in numerous tissues (1, 2). The most significant disease burden occurs within the gastrointestinal tract where, in the absence of endoscopic or surgical intervention, FAP patients have a >90% effective risk of developing colorectal cancer throughout their lifetime (2-4).

FAP patients are also at an increased risk for polyposis within the stomach. The abundance of gastric polyps within FAP patients varies, with some patients developing 100 to 1000’s of lesions and often leading to carpeting of the gastric corpus (5). These polyps predominately arise as fundic gland polyps (FGPs) which are sessile lesions characterized by cystically dilated glands (6-9). FGPs in FAP patients sometimes present with features of low-grade dysplasia, but rarely demonstrate high-grade dysplasia and are typically considered benign (5, 10-12). These polyps are also regionally restricted to the corpus, with gastric polyps in FAP patients only rarely found within the antral region. The rare antral polyps that emerge typically exhibit adenomatous change and have increased potential to progress to cancer in line with colorectal polyps (13).

The mechanisms through which Wnt signaling underscores FGP emergence remain unclear as little is known about how Wnt regulates corpus epithelial homeostasis in humans. Furthermore, it is unknown why FGPs rarely progress to cancer while colorectal cancer in these patients is so prevalent. Although APC mutations have been detected in 7-34% of gastric cancer, FAP patients are only at a 0.5-1.3% lifetime risk of developing gastric cancer (13-17). The mechanism of polyp emergence within the colon would suggest that additional somatic hits to APC, including loss-of-heterozygosity, initiates formation; however, few studies have investigated the mutational landscape of FGPs in FAP patients (18).
The ‘just-right’ hypothesis describes an optimal range of Wnt signaling to drive cellular growth and proliferation in the context of polyposis. This principle has been used to describe the regional distribution of cancer throughout the colon but has not been explored within the stomach (19-23). We have recently demonstrated that corpus organoids derived from patients with no known underlying gastric disease exhibited a lower threshold for Wnt signaling to drive optimal growth relative to subject-matched antral organoids (24). This finding predicts that, at baseline, corpus stem/progenitor cells may be more susceptible to develop hyperplasia upon Wnt activation compared to antral stem cells, but this concept and the subsequent application of the ‘just-right’ hypothesis underscoring FGP development has not been defined.

In this study, we investigated the etiology of FGP formation resulting from germline APC mutations in FAP patients. We established a biobank of patient-matched FGP and surrounding non-polyp gastric tissue samples to conduct comparative analyses of genomic DNA sequencing, mRNA expression analysis, and in vitro organoid growth experiments. We demonstrate that FGPs have increased expression of Wnt target genes relative to their patient-matched non-polyp samples, indicating upregulation of the Wnt signaling pathway. However, additional somatic alterations to APC indicating loss-of-heterozygosity were infrequent, demonstrating that this mechanism does not underscore enhanced Wnt signaling and is not required for polyp emergence. Ultimately, we demonstrate through human FAP organoids and genetic mouse models that heterozygous loss of APC optimally drives corpus proliferation while homozygous loss is not tolerated.
Results

Enhanced Wnt target gene expression in FAP-associated fundic gland polyps

APC mutation activates Wnt signaling through increased translocation of \( \beta \)-catenin to the nucleus resulting from compromised function of the destruction complex (Figure 1A). To investigate the molecular and cellular etiology of gastric FGP formation in FAP patients, we established a biobank of patient-paired FGP (P) and surrounding non-polyp (NP) gastric corpus tissue samples (Figure 1B). From biopsy sample pairs, we extracted genomic DNA, RNA, and/or developed paired organoid cultures for further analyses. Our biobank consisted of samples from 34 individual FAP patients across a spectrum of germline APC mutations and clinical features (Figure 1C, Table 1, Supplementary Table 1).

We first analyzed the gene expression profile of patient-matched P versus NP biopsies to determine whether upregulated Wnt signaling underscored FGP emergence. qPCR analysis of transcript abundance revealed that Wnt target genes LGR5, AXIN2, and CD44 were upregulated in FGP biopsies relative to patient-matched NP tissue (Figures 1D, S1A). Analysis of differentiated cell markers showed that FGP biopsies were deficient in markers for surface cells, which is consistent with studies showing that upregulation of Wnt signaling alters corpus epithelial cell differentiation (Figures 1D, S1B) (24-26). FGP biopsies also showed markedly reduced expression of chief cell markers LIPF, CHIA, PGC, and TNFRSF19 along with a more subdued decrease in the mucous neck cell marker MUC6 (Figures 1D, S1D-E). It is unknown whether these changes in cell marker expression are a consequence of biopsy extraction or due to altered stem/progenitor cell differentiation secondary to Wnt activation in FGPs. Notably, parietal cell markers GIF and H/K ATPase subunit ATP4A were unchanged (Figures 1D, S1C). Analysis of inflammatory markers showed a no difference in CD45 expression, suggesting that FGP formation was not associated with inflammatory cell influx, although some polyps exhibited a pro-inflammatory environment suggested by increased expression of IL8 (Figures 1D, S1F).
Wnt tone is intrinsically elevated in high Wnt FGP-derived organoids

Given our observations that Wnt target gene expression was upregulated in FGPs, we tested whether Wnt signaling is intrinsically elevated in corpus progenitors by generating P- and NP-derived organoids. We passaged FAP patient-derived organoids at least three times before analysis to remove non-epithelial cells and to establish stable self-renewing cultures. Wnt target gene expression was measured in nine patient-matched organoid pairs after growth in normal media containing exogenous Wnt signaling ligands (WNT, RSPO; WR media) and following transition into WNT/RSPO depleted media (WR-Free) (Figure 2A). Comparison of LGR5 mRNA abundance in FAP patient-matched P- and NP-derived organoids revealed variability in intrinsic Wnt tone, with polyp organoids falling into two subgroups. In some patient pairs, such as H62 and H71, we noted either no difference in LGR5 expression between P and NP organoids, or even decreased LGR5 in P organoids grown in WR media (Figure 2B). Other patients, such as H72 and H73, demonstrated enhanced LGR5 expression in P organoids. In all lines, we observed that LGR5 expression was drastically reduced following acute WNT/RSPO withdrawal, thus demonstrating that all lines remained sensitive to exogenous Wnt. Broadening our analysis to include AXIN2 and SP5 demonstrated consistent trends in Wnt target gene expression when normalized to patient-matched non-polyp organoids grown in WR media (Figure 2C). Based on this analysis, we sub-grouped the organoids into High Wnt polyps with enhanced Wnt target gene expression relative to patient-matched non-polyp organoids (H72, H73, H61, H87), and Low Wnt polyps with similar or reduced target gene expression (H76, H71, H62, H82, H75) (Figure 2D).

We further assessed intrinsic Wnt tone by growing organoids for two passages in WR-Free media. Consistent with previous studies reporting that corpus organoids require both exogenous WNT and RSPO to be maintained over time, none of the non-polyp or Low Wnt polyp lines were capable of sustained growth in Wnt-depleted media (25). In contrast, all four High Wnt polyp lines grew long-term in WR-Free media, thus demonstrating long-term Wnt
independent growth (Figure 2E). Therefore, enhanced Wnt target gene expression and growth in Wnt-depleted media suggested an intrinsic increase in Wnt signaling in these FAP polyps.

To test whether High Wnt polyp organoids grow in WR-Free media because they secrete endogenous WNT ligand, we measured growth after treatment with the PORCN inhibitor LGK974, which suppresses Wnt ligand secretion (Figure S2A). We observed that LGK974 treatment had no impact on growth of High Wnt polyp lines H72P, H73P, or H87P (Figure S2B-C). We further observed that neither co-culture of High Wnt organoids growing in WR-Free media with non-polyp organoids, nor feeding non-polyp organoids with conditioned media from these High Wnt lines could rescue non-polyp organoid growth in the absence of exogenous WNT/RSPO (Figure S2D-G). Therefore, we demonstrate through both pharmacologic inhibition and co-culture experiments that High Wnt polyp organoids are sustained long-term in WR-Free media through an intrinsic signaling mechanism rather than by secreting Wnt or other growth factors into their environment.

*Increased growth of High Wnt FGP-derived organoids in reduced Wnt media*

Wnt signaling in the gastrointestinal tract has been proposed to follow a principle known as the ‘just-right’ hypothesis where excessive growth leading to polyposis is driven by an optimal level of Wnt. While too little Wnt signaling induces differentiation, hypermorphic upregulation of Wnt signaling, such as through total loss of APC function, can also be prohibitive to growth. This principle has previously been demonstrated to underscore regional patterns of colorectal cancer emergence in FAP patients where second-hit mutations to APC leading to further dysregulation of Wnt signaling are selected to preserve some residual APC function (20-23, 27). We were interested in whether this principle also holds true for growth of FAP gastric polyp organoids. We hypothesized that High Wnt polyp organoids would have a reduced tolerance for extrinsic Wnt due to elevated intrinsic Wnt signaling, and therefore would exhibit maximal growth at lower concentrations of exogenous Wnt than non-polyp or Low Wnt polyp organoids. To test sensitivity
to extrinsic Wnt, we grew organoids over two passages (12 days total) in either normal (100%) WR or reduced (60%) WR media to define the optimal Wnt environment for growth for each organoid line (Figure 2F). At day 12, organoids were imaged to assess overall appearance, and growth was measured using an ATP-dependent cell viability assay.

Using organoids from patient H87 as an example, we observed that non-polyp organoids grew at similar densities in 100% and 60% WR media (Figure 2G). In contrast, H87 polyp organoids, which are in the High Wnt subgroup, grew denser in 60% WR. Extending this analysis to all nine patient lines, we observed that non-polyp organoids exhibited similar growth in 60% WR media as they did in 100% WR (Figure 2H). While Low Wnt polyp-derived organoids had, on average, no change in growth between the two conditions, polyp organoids from the High Wnt group exhibited significantly enhanced growth in 60% WR. These results align with the ‘just-right’ hypothesis and demonstrate an interplay between intrinsic Wnt tone and extrinsic Wnt signaling for optimal growth.

**Intra-patient variability in FGP-derived organoid growth**

We sought to further investigate the relationship between intrinsic Wnt tone and Wnt sensitivity through an analysis of additional FAP patient-derived organoids. We were also interested in whether individual polyps from the same patient would exhibit polyp-to-polyp variability or similar characteristics, which might suggest genotype-dependent mechanisms. To study this, we expanded our organoid biobank to include multiple independent polyp and non-polyp samples from 17 additional patients (Figures 3A, 1C; Table 1, Supplementary Table 1). Organoids were established by directly embedding minced biopsy tissue in Matrigel, which improved establishment efficiency compared to our previous gland isolation approach. Based on our growth analysis, we established these organoids in 60% WR media to prevent selection bias or suppression of growth in lines with elevated intrinsic Wnt tone. Epithelial outgrowth was observed after 2-4 days in culture, and subsequent passaging resulted in purification of
epithelial organoids (Figure S3A). We noted that most polyp-derived biopsies developed organoids at faster rates than non-polyp biopsies, suggesting that polyps had increased progenitor potential (Figure S3B-C). Organoids were passaged at least three times before analysis to establish pure epithelial cultures.

We evaluated Wnt sensitivity by passaging these FAP organoids into media containing the pharmacologic Wnt activator CHIR99021 (CHIR) to dose-dependently activate Wnt signaling in the absence of exogenous WNT/RSPO ligands (Figure 3B) (24). Organoids were cultured for five days in media containing 0 – 3 µM CHIR with recombinant Noggin (CN Media) to assess Wnt-dependent growth.

In each patient sample set, we observed polyp-to-polyp variability in Wnt sensitivity (Figures 3, S4). For example, from patient H102 we established organoids from six unique fundic gland polyps and three distinct non-polyp regions. Images taken on day 5 of CN media showed that while non-polyp line H102NPα had a positive growth response to increasing Wnt, polyp line H102Pγ was Wnt-averse, with reduced growth in response to increased CHIR (Figure 3C). While all H102 non-polyp lines followed the growth patterns of H102NPα, we observed that some polyp lines, including Pβ, Pγ, and Pε, showed enhanced Wnt-independent growth and reduced growth with increased Wnt signaling (Figure 3D). In contrast, increased CHIR stimulated growth in lines Pα, Pδ, and Pζ, similar to non-polyp lines.

This CHIR growth analysis stratified the expanded patient polyp organoid pool into two subgroups. Regardless of patient (Table 1, Supplementary Table 1), we found that all non-polyp organoids followed a near identical dose-response pattern with growth peaking at ~3 µM CHIR (Figure 3E). One subgroup of polyp organoids (termed Normal-Like or P-N) had a similar growth pattern with increased CHIR leading to increased growth (Figure 3F). The second subgroup (Enhanced or P-E) demonstrated Wnt independence and high sensitivity to increased CHIR (Figure 3G). From 35 polyp lines, 14 (40%) were P-N and 21 (60%) were P-E. While some
patients’ polyps exhibited either P-N or P-E growth characteristics, others exhibited both types (Figure S4). This variability suggests that polyp growth characteristics are not strictly genotype determined, although the small numbers of polyps analyzed from each patient makes it difficult to reach conclusions.

To determine if P-E organoids had higher Wnt signaling tone, we cultured organoids for six days in 60% WR media and measured Wnt target gene expression (Figures 3H-I, S5A). Using H102 organoids as an example, we determined that LGR5 expression was positively correlated with enhanced Wnt-independent growth, suggesting that upregulated Wnt tone corresponded with increased Wnt sensitivity (Figure 3H). Expanding this analysis showed that P-E organoids had significantly upregulated Wnt target gene expression relative to paired non-polyp and P-N organoids, consistent with our previous findings and the ‘just-right’ hypothesis (Figure 3I).

We also analyzed cell differentiation markers in these three organoid types (Figure S5B-E). We and others have shown that Wnt regulates a bimodal axis of differentiation in human and mouse corpus organoids, with low Wnt promoting surface mucous cell differentiation and high Wnt promoting neck and chief cell differentiation (24-26). Analysis of cell marker expression showed that P-E organoids had the lowest expression of surface cell markers MUC5AC, TFF1, and TFF2, consistent with enhanced Wnt signaling (Figure S5B). This aligns with our previous study and confirms that intrinsic Wnt pathway activation directly suppresses surface cell differentiation similar to exposure to exogenous WNT ligand. Interestingly, while our biopsy analysis showed a consistent decrease in chief cell markers across all polyp samples, analysis of P-E organoids demonstrated increased expression of some (LIPF and TRFRSF19) but not all (PGC) chief cell markers (Figure S5D). However, PGC can also localize to human gastric neck cells, and we did not observe alterations in neck cell marker expression (Figure S5C) (28). This finding of increased chief cell marker expression in organoids with increased Wnt pathway activation is consistent with previous studies and confirms the role of Wnt signaling in regulating
the bimodal axis of differentiation within the corpus, with low Wnt driving surface cell
differentiation and high Wnt driving chief cell differentiation (24-26).

The organoid subgroups also exhibited morphological differences. While non-polyp and
normal-like polyp organoids were similar, P-E organoids were significantly thinner (Figure 3J, K).
We speculate that this may be due to the altered differentiation characteristics.

FAP fundic gland polyps develop without requirement for APC loss-of-heterozygosity

We sought to understand the mutational landscape that led to enhanced Wnt signaling in
biopsies and organoids that may underscore FGP formation. One likely mechanism would be
somatic APC mutation leading to loss-of-heterozygosity and further upregulation of the Wnt
signaling pathway as this underscores FAP polyp emergence in the colon. DNA was extracted
from our initial 19 patient biopsies or organoids for genomic analysis of tumor suppressors and
oncogenes using a Qiagen Comprehensive Cancer Gene Panel, which included APC (Table 2).
In each paired patient set (P vs. NP) we confirmed the familial APC mutation. Our analysis
revealed that only one of the 19 patients studied (H85) had a novel somatic loss-of-function
APC mutation (Figure 4A, Table 2). Further intra-patient comparisons revealed that about a third
(6/19) of polyp samples harbored APC-specific copy number variations, although this analysis
did not provide insight into whether the mutant or functional (normal) allele was affected (Figure
4B, Table 2). Looking broadly at the genomic landscape across the target genes included in the
panel, we observed allelic variation that demonstrated chromosomal changes ranging from no
observed copy number variations to extensive (50+) incidences in polyps, suggesting enhanced
chromosomal instability was frequently associated with polyposis (Figure 4C, Table 2).
A low Wnt environment can select for Wnt-activating mutations to sustain organoid growth

We tested whether there was transcriptional silencing of the wild-type APC allele during polyp organoid culture as another mechanism to enhance Wnt tone. APC is hypermethylated in gastric cancer (53%) relative to normal (38%) tissue (29). Although High Wnt polyp organoids from H72, H73, and H87 demonstrated few genomic mutations or copy-number variations, transcriptional silencing of the wild-type allele could explain their elevated Wnt tone. We tested whether High Wnt organoids grown in WR or WR-Free media exhibited loss of the wild-type APC transcript by sequencing mRNA harvested from these three organoid lines at their respective APC familial mutation.

Patients H72 and H73, a parent-child pair sharing a common familial mutation, and patient H87 had inherited four base-pair deletions at c.5826_5829 and c.4782_4785, respectively (Figure 4D, F). Sequencing APC mRNA at the familial mutation site for H72 and H73 polyp-derived organoids grown in WR culture conditions confirmed transcription of both normal and mutant alleles (Figures 4E and S6). This was determined by bifurcation of sequencing products 3’ to the mutation site. However, after growth for three passages in WR-Free media, H72P and H73P organoids only expressed the mutant allele (Figure 4E). In contrast, H87P organoids retained the wild-type APC transcript in both growth conditions (Figure 4G).

To further understand the dynamics of loss of wildtype APC in H73P organoids grown in WR-Free media, we utilized organoids from the earliest available passage post initial biopsy seeding to minimize the influence of genetic drift in culture. H73P organoid RNA was harvested at passage 2 in WR media, and then at passage 3 after six days growth in 100%, 50%, or 0% WR media (Figure S6A). As before, we detected both wild-type and mutant transcripts in WR conditions (Figure S6B). However, after passage, while organoids grown in 100% or 50% WR media maintained expression of both alleles (Figure S6C), growth in WR-Free conditions rapidly led to loss of the wild-type transcript. Thus, although increased Wnt tone in High Wnt organoids
was not associated with silencing of the normal \textit{APC} allele when grown in media containing WNT/RSPO ligands, organoid growth in a WR-Free environment can select for loss of expression of the wildtype \textit{APC} allele.

Following the 'just-right' hypothesis, we hypothesized that the optimal growth of polyp organoids adapted to WR-Free culture conditions would occur in media with minimal exogenous Wnt. We titrated each of the three WR-Free lines to their optimal Wnt signaling environment and determined that optimal growth occurred in 20% WR media (Figure S6D). Ultimately, this establishes a relationship between extrinsic and intrinsic Wnt in accordance with the 'just-right' hypothesis for gastric corpus organoids. As intrinsic Wnt tone is increased through mutation or otherwise, tolerance to extrinsic Wnt is reduced (Figure S6E).

\textit{Mice demonstrate region-specific gastric proliferation in response to Apc mutation}

Our data suggests that genomic \textit{APC} loss-of-heterozygosity is not required for FAP polyp emergence. Further, FAP organoid growth suggests that polyp organoids do not tolerate high levels of extrinsic or pharmacologic Wnt activation in accordance with the 'just-right' hypothesis. These findings, in combination with the low clinical prevalence of cancer progression from FAP polyps, suggest that additional somatic mutations to \textit{APC}, such as \textit{APC}-null, may not be tolerated within the corpus epithelium. Rather, in the corpus, the \textit{APC} heterozygous state may be sufficient to promote growth. We sought to test these concepts in vivo in a mouse FAP model to determine the effect of genomic \textit{Apc} loss on corpus epithelial cell proliferation compared to the antrum where, in FAP patients, infrequent polyps are typically adenomatous.

\textit{Apc\textsuperscript{\textalpha}} mice with \textit{loxP} sites surrounding exon 14 were crossed to \textit{Sox2-CreER\textsuperscript{T2}} mice. \textit{Apc} mutation was induced in adults by treatment with tamoxifen and gastric tissue was harvested from control (\textit{Apc}\textsuperscript{\textomega/\textepsilon}; \textit{Apc}\textsuperscript{0/\textomega}), heterozygous (\textit{Sox2-CreER\textsuperscript{T2}; Apc}\textsuperscript{\textomega/\textepsilon}), and homozygous (\textit{Sox2-CreER\textsuperscript{T2}; Apc}\textsuperscript{0/\textomega}) mice one month later (Figure 5A). We confirmed \textit{Apc} exon 14 deletion in
DNA isolated from corpus and antrum by PCR amplification 48 hours post-tamoxifen (Figure 5B). Further analysis demonstrated that heterozygous Apc deletion was maintained one-month post-deletion in both corpus and antrum, while, in contrast to antrum, homozygous deletion of Apc was poorly maintained in the corpus (Figures 5B, S7).

Further analysis of tissue histology and epithelial cell proliferation also showed stark differences in the cellular response to Apc mutation in the gastric corpus and antrum. While Apc homozygous deletion had no apparent effect on corpus tissue one-month post-tamoxifen (Figure 5), polyp-like growths were observed throughout the antrum (Figure S8). Strikingly, while morphometric analysis of proliferation showed no change in corpus epithelial proliferation in homozygous mice one-month post-TX, we observed a significant (~4x) increase in heterozygous mice (Figure 5C-D). In contrast, there was no observed change in antral proliferation in heterozygous Apc mutant mice, while homozygous Apc mutation resulted in profound hyperproliferation and the formation of polyp-like structures (Figures 5E-F, S8).

We further tested the effect of Apc mutation on mouse gastric organoid growth. Organoids were initiated from gastric glands isolated from control, heterozygous, and homozygous Apc mutant mice 48 hours post-tamoxifen treatment, and organoid size was measured four days later. Consistent with the in vivo findings, heterozygous Apc mutant corpus organoids (Sox2CreERT2; Apcfl/+)) grew larger than control while homozygous Apc deleted corpus organoids (Sox2CreERT2; Apcfl/fl) exhibited diminished growth (Figure 5G). In contrast, antral organoids demonstrated a stepwise growth advantage in response to heterozygous and homozygous Apc deletion (Figure 5H).
Discussion

This study investigated how dysregulation of Wnt signaling in FAP patients leads to abundant yet benign FGPs in the gastric corpus. We initially hypothesized that polyp emergence in the FAP patient corpus was due to somatic mutation resulting in \( APC \) loss-of-heterozygosity. This would align with the general understanding of colorectal polyposis in which a second hit to \( APC \) is considered a requirement and the first step in the mutational cascade inevitably leading to colorectal cancer (18, 30). Surprisingly, we determined that \( APC \) loss-of-heterozygosity is not a requirement for gastric FGP formation in FAP patients as underlying somatic \( APC \) alteration was uncommon. A prior comparative study of the mutational landscape underscoring FGP formation demonstrated that 51% (21/41) of FAP-associated polyps obtained from 17 patients had acquired a somatic \( APC \) gene alteration through either a loss-of-function mutation (15/41) or allelic loss (6/41) resulting in loss-of-heterozygosity (31). While this study did demonstrate a higher proportion of FGPs arising from somatic \( APC \) gene alterations than ours, it aligns with our finding that \( APC \) loss-of-heterozygosity is not a requirement for their emergence.

Despite an absence of second-hit somatic \( APC \) alteration within our FAP patient biobank, we observed that Wnt signaling was generally upregulated in FGP biopsy samples as well as within a subset of our polyp-derived organoids. This underscores a role for Wnt activation in driving polyp formation; however, a precise mechanism driving increased pathway activation within these polyps remains unclear. We explored \( APC \) copy number variation and transcriptional regulation as two mechanisms that could lead to increased Wnt signaling in lieu of genomic mutation. While we did not observe transcriptional silencing of the normal \( APC \) allele in organoids under normal growth conditions, we did observe that some lines were capable of acquiring this phenotype in order to survive within a WNT-free environment. Further, our targeted genomic analysis demonstrated \( APC \) copy number variation within some FGPs (6/19), as well as significant copy number variation across our full gene panel. This finding aligns with previous studies in colorectal cancer cell lines showing that loss of \( APC \) function leads to
chromosomal instability through disrupting its role in microtubule binding (32). We speculate that heterozygous loss of APC confers genetic instability that could sensitize the tissue to polyposis in lieu of a second-hit APC mutation.

Alterations to other key regulators of the Wnt pathway, such as β-catenin, could explain increased Wnt signaling in FAP polyps. While we didn’t observe CTNNB1 mutations within our data set, studies have shown that sporadic FGPs in otherwise healthy patients often contain β-catenin activating mutations, further supporting a role for elevated Wnt signaling in FGP polyp formation (33, 34). Overall, we hypothesize that the mechanisms leading to enhanced intrinsic Wnt signaling within FGPs are likely complex and vary from polyp to polyp as we observed significant variability in Wnt responses across our patient set and even within single patients.

Our biobank of FAP polyp-derived organoids included some without apparent increases in intrinsic Wnt signaling as they exhibited similar gene expression and Wnt tolerance as non-polyp organoids. Notably, these Low Wnt or Normal-Like polyp organoids were derived from a variety of FAP patients without obvious associations with APC genotype or clinical features such as PPI usage, gastric polyp phenotype, or intestinal polyp burden (Table 1, Supplementary Table 1). It is plausible that outgrowth of cells with more normal-like Wnt characteristics could be selected for in vitro because all organoids were established in media containing WNT/RSPO, therefore providing a growth advantage towards cells with more moderate Wnt characteristics. However, primary tissue biopsies also demonstrated heterogeneity in Wnt target gene expression, suggesting that selection in culture does not necessarily underly this variation in intrinsic Wnt tone among our organoid collection. Therefore, additional Wnt-independent mechanisms likely also underscore FGP emergence in FAP patients. A recent study of wildtype murine gastric organoids utilized a genome-scale CRISPR screen to identify genes promoting growth in WNT/RSPO deplete conditions (35). While mutations to Wnt pathway genes such as Apc and Alk were found to support organoid growth by upregulating intrinsic Wnt signaling, this screen also identified genes, including Bclaf3 and Prka, that supported growth through Wnt-
independent mechanisms. Additional mechanisms, such as through environmental factors, may also play a role in the manifestation of FGPs in FAP patients without a requirement for Wnt pathway upregulation.

Regardless of the mechanism by which Wnt is upregulated in FGPs in FAP patients, the same underlying \(\text{APC}\) mutation appears to have strikingly different effects in driving polyposis in the human gastric corpus versus antrum. Wnt signaling has been demonstrated to play a critical role in differentially regulating identity between the two compartments, with high signaling levels being essential for corpus specification during development (36, 37). A recent study from our lab demonstrated that human corpus-derived organoids generated from patients without underlying disease have a reduced threshold for Wnt to induce growth compared to patient-matched antral organoids (24). Combined with the present study, our findings suggest that corpus polyposis in FAP patients may result from an intrinsic heightened sensitivity for Wnt to drive proliferation in corpus progenitor cells. That is, the familial, heterozygous \(\text{APC}\) mutation may prime corpus progenitors to hyperproliferate, while antral progenitors require further second-hit \(\text{APC}\) mutations to induce proliferation, thus underscoring the increased risk of antral adenoma. This aligns with the abundance of FGPs (100’s to 1000’s of lesions) in some FAP patients as there would be no requirement for the rare genomic occurrence of a second \(\text{APC}\) hit. Comparative studies of corpus FGPs to antral polyps in FAP patients would provide further context regarding the underlying genetic mechanisms underscoring emergence and potential for progression to cancer. However, antral polyps are rare in FAP patients and the increased malignant potential of the few that do arise complicates their acquisition for research purposes. Therefore, we were unable to obtain antral polyps during the course of this study to conduct these interesting comparative analyses.

Our theory of regional responses to Wnt tone underscoring polyp emergence was supported by analysis of an FAP mouse model where we showed stark differences in response to \(\text{Apc}\) mutation in the corpus and antrum. We observed that the murine stomach displayed a
similar regional pattern as human FAP, with heterozygous Apc deletion (Apc^{+/−}) promoting corpus proliferation and homozygous deletion (Apc^{−/−}) promoting profound antral hyperplasia while the corpus is spared. Our data showed that the homozygous Apc mutation was not maintained in the corpus, thus suggesting that loss-of-heterozygosity is not tolerated in this gastric region. Previous studies in mice have also showed that homozygous deletion of Apc in differentiated chief cells did not promote corpus hyperplasia (38, 39). Additionally, a long-term study of an Apc_{min/+} mouse model, which closely mimics the FAP condition through a germline Apc mutation, demonstrated that gastric tumors in these mice overwhelmingly arise within the antrum while only rarely occurring in the corpus (40). The majority of these antral tumors were confirmed to be adenomas resulting from Apc loss-of-heterozygosity. Our study contextualizes these findings by demonstrating that Apc^{−/−} cells are either not retained in the corpus epithelium long-term or do not contribute to cellular turnover. Conversely, Apc^{+/−} cells are maintained and contribute to enhanced corpus proliferation. Importantly, another study of heterozygous Apc mutant mice (Lrig1CreER^{T2};Apc^{fl/+}) showed evidence of hyperplasia and increased proliferation in the corpus and antrum 100+ days following recombination (41). Overall, the body of literature in combination with our results suggest that heterozygous loss of Apc in mice mimicking the FAP condition promotes a favorable environment for corpus proliferation while additional Apc gene mutation is not tolerated.

Our findings align with a principle of Wnt signaling known as the ‘just-right’ hypothesis which describes a Goldilocks zone of signaling tone to drive excessive growth in the context of FAP polyposis. Studies exploring this concept have demonstrated that there are regional differences in Wnt signaling tone throughout the small intestine and colon that contribute to region-specific Wnt sensitivity and tumorigenesis (20, 23, 27, 42). We have now expanded this understanding to the stomach by demonstrating that regional differences in Wnt signaling in the corpus versus the antrum may contribute to the emergence of gastric polyps.
The ‘just-right’ hypothesis predicts an upper limit to Wnt signaling that becomes growth prohibitive (22, 43). An intermediate level of Wnt signaling therefore best promotes hyperproliferation and tumorigenesis, with the optimal level differing along the gastrointestinal tract (20, 22, 43). Our study confirms these findings in gastric polyp organoids and demonstrates that increased intrinsic Wnt signaling sensitizes organoids to additional upregulation of the Wnt pathway. This, again, aligns with the concept that somatic APC mutation may not be tolerated within the FAP corpus in vivo as Wnt signaling would be elevated beyond an upper threshold of tolerability. Furthermore, these principles would underscore the benign nature of FGPs relative to polyps of the antrum and colon.

In conclusion, our study translates the ‘just-right’ hypothesis of Wnt signaling to the clinical manifestation of gastric disease caused by pathway dysregulation and develops two unique conclusions related to FGP emergence and pathogenesis in FAP patients. First, we demonstrate that Wnt signaling is predominately upregulated in polyp tissues as well as in polyp-derived organoids and therefore confirm that enhanced intrinsic Wnt signaling is a key characteristic of FGPs. The lack of requirement for a second hit to APC may explain the abundant nature through which FGPs emerge. Second, we establish a definitive relationship between intrinsic and extrinsic Wnt signaling in gastric corpus organoids and demonstrate that additional Wnt signaling in cells with intrinsically upregulated Wnt tone prohibits growth. The corpus environment may therefore selectively prohibit high intrinsic Wnt signaling, such as through APC loss-of-heterozygosity, thus underscoring the benign nature of fundic gland polyps as well as the regionality of their emergence.
Methods

FAP patient biopsy collection and processing

Human gastric tissue biopsies were collected from patients undergoing endoscopy at Michigan Medicine by experienced endoscopists who were familiar with the design and goals of the study. Biopsies were obtained using cold forceps to target areas of polyp tissue and tissue without visible polyps. Biopsies were placed in 15 mL conical tubes containing 5 mL of ice-cold DPBS (Gibco, 14190144) with antibiotic-antimycotic (100 U/mL Pen/Strep + 250 ng/mL Amphotericin B, Gibco, 15240062) for transport to the research lab.

Biopsies were minced into small fragments (~1mm) and divided for nucleotide (DNA or RNA) extraction or organoid formation. Fragments set aside for nucleotide extraction were placed into 1.5 mL Eppendorf tubes and snap-frozen in liquid N₂. Fragments for organoid formation were transferred to 5 mL ice-cold DPBS + antibiotic-antimycotic for subsequent processing. In some instances, minced biopsies were frozen for later organoid development by resuspending in DMEM/F12 (Gibco, 12634010) with 10% DMSO and 10% Fetal Bovine Serum (Sigma, F0926) and frozen in cryovials for long-term storage in liquid N₂ (44).

Establishment and culture of human gastric organoids

Organoids were established from either isolated gastric glands or directly from minced tissue that was embedded in 40 µL Matrigel (Corning, 354234) in a 24-well plate. For the isolated glands approach, 15 mM EDTA (Invitrogen, 15575038) was added to minced biopsies in 5 mL DPBS + antibiotic-antimycotic in 15 mL conical tubes and rocked at 4°C for 1 hour. Tissue fragments were transferred to a new tube containing 8 mL of ice-cold DPBS and gastric glands were released by vortexing at maximum speed for 2 minutes. The suspended gastric glands were transferred to a new tube, pelleted at 600xg for 5 minutes at 4°C, and resuspended in Matrigel for plating. Alternatively, minced tissue was directly embedded in Matrigel, with
epithelial outgrowths emerging within 2-3 days of plating (Figure S3). Organoid lines were established by passaging at least three times before analysis.

Organoids were maintained in WR (WNT, RSPO, Noggin), WR-Free (Noggin), or CN (CHIR99021, Noggin) media as specified. 100% WR media was generated with 50% L-WRN conditioned media (University of Michigan Translational Tissue Modeling Laboratory), 10% Fetal Bovine Serum (Sigma, F0926), 37% DMEM/F12 (Gibco, 12634010), 2mM GlutaMAX (Gibco, 35050061), Antibiotic-Antimycotic (100 U/mL Pen/Strep + 250 ng/mL Amphotericin B, Gibco, 15240062), 10 µM Y-27632 (Tocris, 1254), 10 µM SB431542 (Tocris, 1614), and 50 µg/mL Gentamycin (Gibco, 15750060). WR-Free media consisted of 20% Fetal Bovine Serum, 77% DMEM/F12, GlutaMAX, Antibiotic-Antimycotic, Y-27632, SB431532, and Gentamycin in the same concentrations as above, and was supplemented with 100 nM recombinant Noggin (R&D, 6057-NG). For WR media formulations, normal (100%) WR and WR-Free media were mixed at appropriate ratios to obtain the desired final WR concentration. For CN media, CHIR99021 (CHIR, Tocris, 4423) was added to WR-Free media to obtain the final concentration as specified. DMSO added through stock solutions of CHIR and SB431542 was maintained at a final concentration of 0.3% in CN Media.

Organoid cultures were maintained by replenishing with fresh media every two days and passaging every six days. To passage organoids, media was aspirated and Matrigel patties containing organoids were overlaid with 500 µL of cold DPBS, mechanically disrupted through scraping with a P1000 pipet tip and transferred to a 1.5 mL Eppendorf tube. Organoids were pelleted at 250xg for 5 minutes at 4°C, resuspended in 500 µL of TrypLE Express (Gibco, 12604013), and incubated at 37°C for 10 minutes. Organoids were vigorously pipetted approximately 40 times with a P1000 to dissociate into single cells. 700 µL of cold DPBS was added, cells were pelleted at 250xg for 5 minutes at 4°C, resuspended in 30-100 µL of DMEM, and cell concentration was quantified using a hemocytometer. For all experiments, cells were
plated at a density of 300 cells/µL of Matrigel patty. Matrigel patties were given 30-45 minutes to solidify at 37°C, then overlayed with the appropriate warmed media.

**DNA Library Preparation**

DNA was extracted from FAP patient biopsies using the Qiagen Blood & Tissue DNA kit (Qiagen #69504), with sample elution in 100-200 µL Buffer TE. For DNA extraction from paired non-polyp and polyp organoid samples, culture media was aspirated from each Matrigel patty (3 wells pooled/sample), organoids were suspended in DPBS, pelleted at 300xg for 5 min, resuspended in 200 µL DPBS, and DNA extracted.

For preparation of genomic DNA libraries, samples were indexed using the QIAseq 96-Index I Set A kit (Qiagen #333727) and libraries were constructed for sequencing using the QIAseq targeted Human Comprehensive Cancer Panel (Qiagen #333515, catalog #DHS-3501Z-96). NextGen DNA sequencing was performed using a HiSeq 4000 (Illumina), with sequencing and analysis conducted in collaboration with the University of Michigan Advanced Genomics Core.

**RNA extraction, qPCR analysis, and mRNA sequencing**

RNA was isolated using the Qiagen Mini Kit (Qiagen, 74106) according to the manufacturer’s instructions. Frozen minced tissue from biopsies was transferred to RLT Buffer + 1% βME and homogenized, and centrifuged at 14,000xg for 3 minutes to pellet particulates. Supernatant was collected, transferred to a new tube, and mixed with an equal volume of 70% EtOH. For organoid RNA extraction, organoids were dispersed to single cells using TrypLE, washed, resuspended in RLT Buffer + 1% βME and vortexed at max speed for 30 seconds before RNA extraction.

cDNA was synthesized from 250ng RNA using the iScript™ cDNA Synthesis Kit (BioRad, 1708891). qPCR reactions used the iTaq™ Universal SYBR Green Supermix (BioRad,
1725124) and respective primers (Supplementary Table 2). qRT-PCR was performed with samples in triplicates, and average cycle threshold values were quantified relative to the reference mRNA ACTB or HPRT using the ΔΔCT method to determine mRNA abundance.

For APC mRNA sequencing, RNA was extracted from organoids, cDNA was synthesized, and qPCR reactions were performed using primers encompassing the APC mutation site (Supplementary Table 3). PCR products were purified using the QIAquick PCR Purification Kit according to the manufacturer’s instructions, sent to Eurofins Genomics (Louisville, KY) for sequencing, and sequences were analyzed using SnapGene (San Diego, CA) software.

**Co-culture and conditioned media experiments**

Polyp organoids from lines H72, H73, and H87, as well as non-polyp organoids from H87, were dispersed to single cells. For co-culture experiments, single cells from polyp and non-polyp organoids were plated in independent 10 µL patties of Matrigel at a density of 3,000 cells/patty within the same well of a 24-well plate, taking care to ensure patties did not touch. For conditioned media experiments, the three High Wnt organoid lines were each plated in independent 10 µL patties of Matrigel at a density of 3,000 cells/patty within the same well of a 24-well plate. Cells were overlayed with WR-Free media. On day 4, media from High Wnt polyp organoids was transferred to patties containing freshly passaged single cells from non-polyp organoids. WR-Free media was replenished, and this was repeated every two days for a total of six days.

**Human organoid growth experiments**

For Wnt titration experiments, organoids were dispersed to single cells, plated into triplicate wells of a 24-well plate in 40 µL patties of Matrigel at a density of 12,000 cells/well, and overlayed with the appropriate media. On day 6, triplicate wells of each media condition were
consolidated, organoids were dispersed to single cells, and cells were resuspended at the same split ratio across all conditions. The concentration of cells in 100% WR was calculated, and cells from each condition were plated at the split ratio calculated to reach 12,000 cells/well in 40 µL Matrigel patties for the 100% WR condition. For measurement of growth, media was aspirated, and patties were overlayed with a room temperature 50:50 mixture of DMEM (200 µL) and Cell-Titer-Glo 3D (200 µL) (Promega, G9681). Plates were incubated for 30 minutes at room temperature in the dark, the Matrigel patty was broken down by rapid pipetting, and the total contents of each well were transferred to individual wells of a white, opaque 96-well plate. Luminescence was measured using a plate reader with an integration time of 500ms.

For CHIR growth experiments, organoids were dispersed to single cells and plated into a 96-well round-bottom plate in 5 µL patties of Matrigel at a density of 1,500 cells/well. For measurement of growth, media was aspirated and 150 µL of a room temperature 50:50 mixture of DMEM (75 µL) and CellTiter-Glo 3D (75 µL) (Promega, G9681) was added to each well. Plates were incubated for 30 minutes at room temperature in the dark, the Matrigel patty was broken down by rapid pipetting, and the total contents of each well were transferred to individual wells of a white, opaque 96-well plate. Luminescence was measured using a plate reader with an integration time of 500ms.

Mouse Experiments

Adult mice of both sexes 2-3 months old were housed under specific pathogen-free conditions. Sox2-CreERT² (Jackson Labs #017593)(45) and Apc580floxed (Apc⁰)(46) mice have been previously described. Apc⁰ mice contain a floxed exon 14, which upon Cre-mediated deletion encodes an APC protein truncated at codon 580. Apc⁰ mice were on a 129/SvJae background and Sox2-CreERT² mice were on a mixed C57BL/6 x 129/SvJae background. Sox2-CreERT² and Apc⁰ mice were bred to generate the following genotypes: Sox2CreERT²; Apc⁰/+
(heterozygous), Sox2CreERT2; Apc\^{\text{fl/\text{fl}}\text{}} (homozygous), and controls (Apc\^{\text{fl/\text{n}}\text{}} and Apc\^{\text{fl/\text{fl}}\text{}}, or Sox2-CreERT2).

To induce Cre-mediated recombination of the floxed Apc allele, mice were treated with 100 mg/kg tamoxifen (TX) via intraperitoneal injection once daily for 5 days. For analysis of Apc recombination, full thickness tissue was homogenized, and DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, 69504) per the manufacturer’s instructions. Recombination was analyzed via PCR amplification using the following primers(46): P3: 5′-GTT CTG TAT CAT GGA AAG ATA GGT GGT C-3′, P4: 5′-CAC TCA AAA CGC TTT TGA GGG TTG ATT C-3′, P5: 5′-GAG TAC GGG GTC TCT GTC TCA GTG AA-3′. P3 and P4 generate a 314 bp product encompassing the unrecombined loxP site, while P3 and P5 generate a 258 bp product encompassing the recombined region.

For analysis of proliferation, mice were injected with 5-ethynyl-2′-deoxyuridine (EdU, 25 mg/kg, Invitrogen, A10044) two hours prior to tissue collection. For morphometric quantification of EdU incorporation, the entire length of the corpus and antrum for each animal was imaged (n = 3–9 animals per group) and blinded cell counts were normalized to epithelial tissue length (μm) using ImageJ software (National Institutes of Health, Bethesda, MD).

**Analysis of murine gastric organoids**

Mouse gastric organoids were established from corpus and antrum tissue collected from Sox2-CreERT2 (control), Sox2-CreERT2; Apc\^{\text{fl/\text{n}}\text{}} (heterozygous), and Sox2-CreERT2; Apc\^{\text{fl/\text{fl}}\text{}} (homozygous) mice 48 hours post final TX injection. Gastric tissue was washed in ice-cold DPBS and minced into 2-3 mm fragments. Tissue fragments were transferred to DPBS containing 8 mM EDTA and rocked at 4 °C for 1 hour. To isolate glands, tissue fragments were transferred to a fresh DPBS solution containing 10 mM EDTA and rocked at 4 °C for 2 hours. During the last 10 minutes of incubation, samples were placed on ice to allow glands to settle,
and the EDTA-DPBS solution was replaced with DPBS and gently mixed 3-5 times using an
FBS-coated p1000 tip with the opening enlarged to prevent breaking or sticking of isolated
glands. Once the mixture was cloudy, glands were transferred to a 1.5 mL Eppendorf tube and
centrifuged at 150xg for 10 minutes at 4°C. After aspirating the supernatant, glands were
resuspended in 40 µL Matrigel and plated. Murine gastric organoids were maintained in culture
using the same procedures outlined for human organoid growth, but without the TGF-β inhibitor
SB431542.

For size measurement, mouse organoids were imaged at day 4 of culture using an
Olympus stereomicroscope at 1.7X magnification. The area of growth was calculated from
digital images using the Orgaquant software (47). Three technical replicates were used for each
group and were pooled for analysis, with >170 organoids measured from each condition.

Statistics
GraphPad Prism (version 9.0, Software, San Diego, CA) was used for statistical analysis.
Student’s t test (2-tailed) was used to compare two groups. For comparison of 3 or more
groups, a One-Way Analysis of Variance (ANOVA) was used followed by a Tukey post-hoc test.
*p<0.05, **p<0.005, and ***p<0.001 were used to denote significance.

Study Approval
Collection of human tissue was conducted under Institutional Review Board-approved protocol
(HUM00102771) at the University of Michigan. Written informed consent was provided by
individual patients prior to collection of biopsies. Mouse studies were conducted under
University of Michigan Institutional Animal Care & Use Committee (IACUC) approved protocols
(PRO00010803).

Data Availability
Underlying data, including annotated sequencing data, are available in the corresponding supporting data values file. Organoid lines are available upon reasonable request to the corresponding author.
Acknowledgements

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Author Contributions

K.P.M., E.D., T.M.K., E.S.H., and L.C.S. were responsible for designing research studies.

K.P.M., E.D., and T.M.K. conducted experiments and acquired data. K.P.M., E.D., T.M.K., and L.C.S. were responsible for analyzing data. D.K.T. and E.M.S. were responsible for coordinating human biopsies and providing clinical information. K.P.M. and L.C.S. were responsible for writing the manuscript and all other authors read and provided comments on the manuscript.
References


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* Repeat sample collection from indicated patient. **Age listed to nearest decade.
Table 2: Qiagen Comprehensive Cancer Panel genomic sequencing results

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<td>Biopsy</td>
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<td>Deletion</td>
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<tr>
<td>H81</td>
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<tr>
<td>H82</td>
<td>Organoids</td>
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<td>-</td>
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<tr>
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<td>Biopsy</td>
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<td>c.263delG; p.Ser89fs</td>
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<td>16</td>
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<td>H93</td>
<td>Biopsy</td>
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CNV: copy number variation; - : no changes detected
Figure 1: Fundic gland polyp biopsy samples from FAP patients have increased Wnt target gene expression. A) Schematic of Wnt signaling in Wnt OFF, Wnt ON, and in FAP patients with mutated APC (APC*) exhibiting nuclear localization of β-catenin in the absence of Wnt ligand. B) Biopsies from paired fundic gland polyps (P) and surrounding non-polyp (NP) corpus tissue were collected to establish an FAP patient biobank of RNA, DNA, and organoids. C) Schematic of the APC protein labeled with specific familial mutation sites for the patients in our biobank. Patients with established polyp and non-polyp organoids are designated. Only patients with known germline mutations are included on this schematic (see Table 1). D) Relative mRNA abundance of select genes in FAP patient biopsies. qRT-PCR analysis of Wnt target, cell marker, and inflammation-related transcripts, with HPRT used as an internal reference transcript. Data are displayed as Log(2) fold change (error bars minimum to maximum values) relative to patient-matched non-polyp tissue (n_{non-polyp} = 20-25 biopsies, n_{polyp} = 21-30 biopsies). Statistical analysis by unpaired parametric t test (*p<0.05, **p<0.005, ***p<0.001; see Figure S1).
**A** FAP Organoids

Non-Polyp (NP)  Polyp (P)

**Wnt Target Gene Expression**

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**B** LGR5 mRNA Abundance

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<tr>
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<td>***</td>
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**C** Wnt Target Gene Expression

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<tr>
<td>H87</td>
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</table>

**D** LGR5<sub>p</sub>/LGR5<sub>NP</sub>

<table>
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<tr>
<th>Time</th>
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<tr>
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**E** Long-Term Wnt-Independent Growth

**F** Wnt Titration

Non-Polyp (Low Wnt)  Polyp (Low Wnt)  Polyp (High Wnt)

**G** H87 Organoids Day 12

<table>
<thead>
<tr>
<th>Condition</th>
<th>Field A</th>
<th>Field B</th>
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<tr>
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<tr>
<td>Polyp</td>
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<tr>
<td>100% WR</td>
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<td>60% WR</td>
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**H** Growth in 60% WR

<table>
<thead>
<tr>
<th>Condition</th>
<th>Growth to 100% WR Media</th>
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<tbody>
<tr>
<td>NP</td>
<td></td>
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<tr>
<td>P (Low)</td>
<td></td>
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<tr>
<td>P (High)</td>
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</table>
Figure 2: Enhanced Wnt signaling in a subset of FAP gastric polyp-derived organoids. A) Wnt target gene expression was measured in patient-matched polyp (P)/non-polyp (NP) organoid pairs following six days of growth in WR media or following four days of growth in WR and two days of growth in WR-Free media. B) LGR5 mRNA abundance in FAP organoids grown in WR or WR-Free media. Data shown as mean ± SD mRNA abundance relative to the reference gene ACTB (n = 3 individual wells; *p<0.05, **p<0.005, ***p<0.001 by one-way ANOVA with Tukey’s multiple-comparison test). C) Heatmap of relative Wnt target gene expression in polyp organoids. Data are shown as mean fold-change relative to the expression of each target gene in matched non-polyp organoids grown in WR media for six days. D) Heatmap of LGR5 mRNA expression in polyp organoids, shown as mean fold-change relative to patient-matched non-polyp organoids and ordered from highest to lowest expression. High Wnt and Low Wnt denote the classification of polyp-derived lines with increased or similar/decreased LGR5 gene expression. E) Images of polyp organoids grown for two passages (12 days) in WR-Free media, demonstrating Wnt-independent growth (size bars = 100 µm). F) Growth of organoids cultured for 12 days in 100% or 60% WR media was measured through ATP-dependent luminescence. G) Images of H87 organoids at day 12 following growth in 100% or 60% WR (size bars = 200 µm). H) Relative growth of non-polyp and polyp organoids clustered by Wnt target gene expression characteristics (High Wnt and Low Wnt) in 60% compared to 100% WR media. Each point represents an individual organoid line shown as the average of triplicate wells. Data shown as mean ± SD of the organoids in that group (n=4-9 individual organoid lines; *p<0.05, **p<0.005 by one-way ANOVA with Tukey’s multiple-comparison test).
A. FAP Patient

B. CHIR99021 Growth and Relative Growth of FAP Polyp and Non-Polyp Organoids over 5 days.

C. CHIR Concentration (µM) for H102 and relative growth.

D. H102 CHIR Growth 5 Days with CHIR Concentration (µM).

E. FAP Non-Polyp

F. FAP Polyp - Normal-Like

G. FAP Polyp - Enhanced

H. LGR5 vs 0 µM CHIR Growth with Relative Growth (µM CHIR). R² = 0.5771

I. Wnt Target Genes with Relative mRNA Abundance.

J. Organoid Thickness with Organoid Morphology.

K. Organoid Morphology with H102P, H105P, and H110NP.
Figure 3: Intrinsic Wnt tone patterns intra-patient FGP variability. A) Polyp (P) and non-polyp (NP) biopsies were obtained to establish multiple polyp-derived organoids from each FAP patient. B) Organoids were grown in CN Media with varying concentrations of CHIR99021 (CHIR) and growth was measured through ATP-dependent luminescence on day 5. C) Representative images of H102NPα and H102Py following culture in 0-3 µM CHIR (size bars = 100 µm). D) Relative growth of H102 organoid lines as a function of CHIR concentration. Growth was normalized to the maximal growth observed for each line, with data shown as mean ± SD of triplicate wells. E-G) Relative growth of organoid lines from 12 FAP patients plotted as a function of CHIR concentration. Individual organoid lines are shown in grey, and the average of all lines is shown in color. E) Non-polyp organoid lines (blue, n = 23 lines). F) Polyp organoid lines demonstrating normal-like growth (pink, n = 14 lines). G) Polyp organoid lines demonstrating enhanced Wnt-independent growth (purple, n = 21 lines). H) Relative growth of H102 organoid lines after five days growth in 0 µM CHIR (WR-Free media) versus LGR5 mRNA abundance after six days growth in 60% WR. The trendline was calculated via linear regression analysis. I) Relative mRNA abundance of Wnt target genes in FAP organoids after six days growth in 60% WR. Data shown as mean ± SD fold-change relative to patient-matched non-polyp. NP: n = 19; P-N: n = 11; P-E: n = 13 (*p<0.05, **p<0.005 by one-way ANOVA with Tukey’s multiple-comparison test). J) Average NP, P-N, or P-E organoid thickness after six days growth in 60% WR (***p<0.001). K) Representative images of non-polyp, normal-like polyp, and enhanced polyp organoids after six days growth in 60% WR (size bars = 50 µm).
Figure 4: Infrequent somatic APC mutation in FAP patient FGPs. A) Targeted sequencing of 19 FAP patient polyp and non-polyp samples using a Qiagen Comprehensive Target Cancer Panel detected one patient (H85) polyp with a novel somatic APC loss-of-function mutation. B) Six patients, including H85, demonstrated APC specific copy number variation (duplication or deletion) in their sequenced polyp DNA. C) Copy number variations detected through sequencing across the panel of 283 target genes by patient: None = 0 CNVs (4/19), Few = 1-4 (9/19), Moderate = 5-25 (3/19), Extensive = >26+ (4/19). D) APC mRNA sequence of normal and mutant allele in patients H72 and H73 at the familial mutation site (c.5826_5829). The highlighted CAGA sequence is deleted in the germline mutant APC allele. E) Chromatogram of sequenced APC cDNA harvested from H72 polyp organoids grown in 100% WR media, H72 polyp organoids grown for 2+ passages in WR-Free media, and H73 polyp organoids grown for 3+ passages in WR-Free media, depicting 7 bp 5’ of the mutation site and 6 bp 3’ of the mutation site. The blue underlined sequence aligned with the wild-type sequence. The red underlined sequence aligned with the mutant sequence. F) APC mRNA sequence of the wild-type and mutant allele in patient H87 at the familial mutation site (c.4782_4785). The highlighted AGCC sequence is deleted in the germline mutant APC allele. G) Chromatogram of sequenced APC cDNA harvested from H87 polyp organoids grown either in WR media or for 2+ passages in WR-Free media. The blue underlined sequence aligned with the wild-type sequence. The red underlined sequence aligned with the mutant sequence.
**Sox2-CreERT2;Apc<sup>fl/+</sup> or Sox2-CreERT2;Apc<sup>fl/fl</sup>**

**Corpus (1 month)**

- **EdU** cells/µM
  - **Control fl/+ fl/fl**
  - **0.00**
  - **0.05**
  - **0.10**
  - **0.15**
  - **0.20**
  - **0.25**

**Antrum (1 month)**

- **EdU** cells/µM
  - **Control fl/+ fl/fl**
  - **0.00**
  - **0.05**
  - **0.10**
  - **0.15**
  - **0.20**
  - **0.25**

**314 bp (fl)**

**258 bp (∆)**
Figure 5: Gastric region-specific proliferation in FAP mouse model. A) Adult Sox2CreERT2; Apc^fl/+ (heterozygous), Sox2CreERT2; Apc^fl/fl (homozygous), and control (Apc^f+/+ or Apc^fl/fl) mice were treated with tamoxifen (TX) to delete Apc exon 14, and tissue was harvested one month later. B) Agarose gel showing PCR products amplified from genomic DNA with primers flanking a loxP site in the Apc gene to identify either the unrecombined allele (fl, 314bp) or recombinant allele (Δ, 258 bp). DNA was isolated from full thickness corpus (C) or antral (A) tissue from control (Apc^fl/fl) mice one-month post-TX, or from homozygous Sox2CreERT2; Apc^fl/fl mice either 48 hours or one-month post-TX. C, E) Representative images of corpus (C) and antral (E) tissue from control, Sox2CreERT2; Apc^fl/+ , and Sox2CreERT2; Apc^fl/fl mice one-month post-TX stained for EdU (green) to mark proliferating cells (size bar = 100 µm). D, F) Morphometric quantification of proliferating EdU+ cells per µm of corpus (D) or antral (F) tissue in control, heterozygous (fl/+), and homozygous (fl/fl) mice one-month post-TX. Data are presented as mean ± SEM (n=3-9 mice per group, ***p<0.001 by one-way ANOVA with Tukey’s multiple comparison test). G, H) Size and representative images of corpus (G) or antral (H) organoids derived from control (Sox2CreERT2), heterozygous (fl/+), and homozygous (fl/fl) mice. Data are presented as median ± interquartile range, with error bars representing minimum and maximum values (**p<0.05, ***p<0.005 by one-way ANOVA with Tukey’s multiple-comparison test). Sox2CreERT2 (n= 3 mice; 208 corpus organoids; 217 antrum organoids), Sox2-CreERT2; Apc^fl/+ (n=3 mice; 171 corpus organoids; 256 antrum organoids) and Sox2-CreERT2; Apc^fl/fl (n=3 mice; 218 corpus organoids; 278 antrum organoids).