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Intestinal deletion of *Pofut1* in the mouse inactivates Notch signaling and causes entero-colitis

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Abstract

Background & Aims—Notch downstream targets are fundamental to intestinal cell lineage commitment and are suggested as therapeutic targets for colon cancer cells. However, the role of endogenous Notch signaling through receptor-ligand interaction, and effects of its longer-term down-regulation on intestinal homeostasis, are unclear.

Methods—To address these issues, the gene encoding protein *O*-fucosyltransferase 1, an enzyme required for the Notch ligand binding and thus activation of all Notch receptors, was deleted in the mouse intestinal and colonic epithelium, through Villin-Cre-mediated recombination.

Results—*Pofut1* deletion inactivated Notch signaling giving rise to smaller but viable mice. These mice exhibited a large increase in all intestinal secretory cell lineages which accumulated in the crypts, resulting in crypt hyperplasia. Although proliferating cells were largely reduced in the colon, the transit amplifying compartment was maintained in the upper crypts of the intestinal mucosa. By 9 months, these perturbations in cell maturation altered mucus-associated gut microbiota and caused chronic intestinal inflammation, with evidence for bacterial translocation to the mesenteric lymph nodes, macrophage and T lymphocyte infiltration, and Th1/Th17 immune response. Dysplastic foci were also observed in *Pofut1* deficient small intestine with occasional progression to tumor formation.

Conclusions—Mucus hypersecretion upon *Pofut1* inactivation is accompanied by alteration of the mucus-associated flora, which likely contributes to the development of entero-colitis. Therefore, these data identify important potential complications in strategies to target Notch signaling in therapeutic approaches to colon cancer.

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Introduction

Notch signaling is a highly evolutionarily conserved network that orchestrates cell fate determination, involving regulation of proliferation, migration, differentiation and cell death in organisms ranging from insects to humans. In mammals, Notch signaling is mediated by four Notch genes (*Notch1* through *Notch4*) and five cell-membrane-associated ligands (*Jag1*, *Jag2*, *Dll1*, *Dll3* and *Dll4*). Upon ligand binding on neighboring cells, Notch receptors undergo γ -secretase mediated proteolytic cleavage, which releases the Notch intracellular domain (NICD) from the membrane 1. NICD subsequently translocates to the nucleus and forms a complex with the DNA-binding factor RBP-J κ which upregulates the transcription of target genes², including members of the hairy and enhancer of split (HES) and hairy related transcription factors (HRT). In particular, NICD stimulates expression of HES1³, which represses the activity of other bHLH transcription factors, including MATH1 (or human homolog Hath1)⁴⁻⁵.

Gain-of-function approaches have shown that intestinal crypt progenitors cease to mature towards secretory cell types and continue to proliferate upon constitutive Notch1 activation⁶⁻⁷. Consistent with this, conditional inactivation of RBP-J κ signaling drives cellular differentiation towards goblet and Paneth cell lineages in the mouse intestine⁸. As Notch signaling appeared to be a key, albeit redundant, pathway in crypt homeostasis^{5, 6, 8-11}, its activation was reported indirectly by investigating expression of Notch target genes in human and mouse colorectal and intestinal tumors respectively^{8, 12}. In this context, inactivation of the Notch pathway by use of a γ -secretase inhibitor (GSI), can drive a small percentage of intestinal tumor cells in the Apc^{Min} mice to differentiate and undergo growth arrest, providing proof of principle that this pathway is a potential target for therapeutic approaches to colon cancer⁸.

However, important questions remain unanswered. First, what is the requirement for endogenous Notch/ligand interaction in regulation of intestinal homeostasis? Although the function of HES1, MATH1 and RBP-J κ , downstream components of the Notch pathway, have been addressed by loss of function studies^{5, 8, 9, 13}, these models may not reflect the complex signaling triggered by the different combinations of Notch ligand/receptor interaction¹⁴ along the gut. Second, with regard to the potential use of GSI as a therapeutic approach in colon cancer, what are the longer-term consequences of disruption of intestinal homeostasis by targeting Notch signaling?

To address these issues, we adopted the strategy of blocking all Notch/ligand interactions by inactivating the expression of protein O-fucosyltransferase 1 (*Pofut1*) specifically in intestinal and colonic epithelial cells in the mouse. The rationale for this approach is that Notch receptors require the addition of O-fucose by POFUT1 to conserved Ser or Thr residues in epidermal growth factor-like repeats of their extracellular domain in order to signal through Delta-like and Jagged ligands¹⁵⁻¹⁹. We report that intestinal *Pofut1* deficiency dramatically inhibits Notch signaling, and promotes differentiation of intestinal proliferating progenitors to secretory cell fates. By 9 months, the disruption in intestinal homeostasis gives rise to extensive inflammation throughout the small and large intestine linked to alterations in the gut microbial population, with evidence for development of dysplasia and progression to tumor formation.

Materials and Methods

Animals and Tissue preparation

Pofut1^{F/F} mice¹⁸ were on a mixed 129/C57BL/6 background. Transgenic Tg(Vil-cre)^{997Gum} mice expressing Cre recombinase under control of the mouse villin 1 promoter

20 were purchased from the Jackson Laboratory. Breeding generated mice that were homozygous for the *Pofut1* floxed allele and that harbored a Villin-Cre transgene. Mice were genotyped by PCR analysis as previously described 18, 20. All protocols were approved by the Animal Care and Use Committee at Montefiore Medical Center and the Albert Einstein College of Medicine. Small and large intestine were removed immediately after sacrifice of 4 or 36 week old mice, opened longitudinally and inspected under a dissecting microscope.

Isolation and fractionation of epithelial cells

The small and large intestine were dissected, everted, filled to distension with PBS, and incubated with shaking at 37°C in 1.5 mM EDTA buffer. The sequential isolation of intestinal and colonic epithelial cells along the crypt-villus axis (CVA) was performed as previously described and validated 21, 22. Resulting fractions of dissociated epithelial cells were harvested by centrifugation at 1500 rpm at 4°C for 5 min, cell pellets were snap frozen in liquid nitrogen, and stored at -80°C.

Immunohistochemistry and histological scoring

Intestinal and colonic tissues were fixed overnight in 10% neutral buffered formalin, paraffin-embedded and sectioned at 5 µm. After dewaxing and hydration, sections were pretreated with 3% H₂O₂ for 10 min at room temperature and incubation of antibodies was performed overnight in BSA/PBS at 4°C. Antigen retrieval was achieved by boiling in 10mM citrate buffer pH 6.0 (30 min) for all antibodies, except anti-BrdU, which used 0.1% trypsin (20 min at 37°C). HRP-conjugated secondary antibodies were detected with the diaminobenzidine peroxidase substrate kit (Vector Laboratories). For assay of alkaline phosphatase activity, the substrate (Vector red; Vector Laboratories) was applied to the sections for 10 min.

Histological scores were assigned as follows: 0 = no significant change; 1 = minimal to mild change; 2 = moderate change, and 3 = severe change. This scoring system was applied to: crypt hyperplasia (elongation of the crypts, resulting in mucosa thickening), crypt dilation (crypt lumen widening associated with luminal mucous), inflammatory infiltrates into the lamina propria of the mucosa (evaluated by cell type and number). Moderate increases of infiltrates resulted in the separation of glands by inflammatory cells, more notable in marked inflammation, and associated with some crypt loss.

Antibodies and Western blot analysis

Protein extracts were prepared by homogenization of cell pellets in RIPA lysis buffer at 4°C, centrifugation at 15,000g for 15 min at 4°C, and supernatants were collected for Western blot analysis. Protein concentration was quantified using the Bradford assay (Bio-Rad Laboratories). Solubilized proteins (50–200 µg) were separated by electrophoresis on SDS-PAGE gels, transferred onto activated PVDF membranes and subjected to immunoblot analysis. Antibodies were then used according to the manufacturer's protocols. Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected by chemiluminescence. Equal protein loading was verified by reprobing membranes for β-actin.

RNA isolation and quantitation of steady-state mRNA by Real time PCR

Total RNA was extracted from frozen tissue cell pellets with Trizol reagent (Invitrogen). First-strand cDNA was synthesized from 1 to 5µg total RNA with 200 units of reverse transcriptase using the Superscript II kit (Invitrogen) according to the manufacturer's recommendations.

cDNA was amplified using SYBR Green PCR Master Mix, and the ABI PRISM 7900HT Sequence Detection System Real Time PCR system (Applied Biosystems). Primer sequences (available upon request) were supplied by Sigma-Genosys. Murine actin was used as an internal reference. For each PCR product, there was a single dissociation curve, confirming specificity of the amplification. All reactions were carried out in duplicate. A standard curve was generated by similar analysis of 5-fold serial dilutions of a standard template DNA. Relative values for each PCR product were expressed in arbitrary units (AU) as a ratio of the target transcript normalized to mouse *actin*.

Whole colon culture

1.5cm of colonic flat mucosa was washed in cold PBS supplemented with penicillin and streptomycin, then cut into small pieces and maintained in culture for 24h at 37°C in 2ml of serum-free RPMI medium containing BSA (0.01%) and antibiotics (200g/ml penicillin, 200U/ml streptomycin). Before subsequent cytokine profile analysis, samples were centrifuged at 5000g for 5 minutes at 4°C, supernatants were collected and stored at -80°C

Bacterial cultures

Mesenteric lymph nodes (MLN) and colonic mucosa were removed under sterile conditions, prepared with a sterile grinder and then plated onto either Trypticase soy agar with 5% sheep's blood agar, MacConkey agar, or CDC anaerobes blood agar. Cultures were incubated at 35°C and examined at 24-h intervals for 2 days. Bacterial growth on the agar plates was quantified as the number of colony forming units per µg of tissue.

Results

Intestinal deletion of *Pofut1* induces significant weight loss and Notch signaling inactivation

We first investigated the distribution of *Pofut1* in the normal adult mouse intestinal and colonic epithelium by real time PCR. Fractionation of intestinal epithelial cells along the CVA revealed that fraction 4 was enriched in *Sox9*-expressing progenitor cells from the crypts whereas fraction 1 mainly contained *Fabp2*-expressing differentiated cells from the top of the villi (Fig 1A). *Pofut1* expression was upregulated by more than 4-fold in the crypt compartment and thus displayed a similar expression pattern as Notch receptors and ligands (Fig S1). In the colon, *Sox9* expression was enhanced in fraction 4, although there was a modest, not significant increase in *Pofut1* in the crypt (Fig 1B). Whereas the complex distribution of Notch receptors and ligands suggested specific roles for different Notch-receptor complexes along the gut cephalo-caudal axis (Fig S2), the expression of *Pofut1* was constant along this same axis (data not shown), consistent with its universal role in providing the necessary fucosylation of all Notch receptors.

To achieve inactivation of *Pofut1* in the intestinal and colonic epithelium, *Pofut1*^{F/F} mice 18 were crossed with a constitutive Tg(Vil-cre)997Gum in which Cre recombinase expression was driven by the villin promoter 20. The resulting *Pofut1*^{F/F}:Villin-Cre progeny were obtained in the expected Mendelian ratio. Efficient recombination was demonstrated by the presence of only the deleted form of the floxed allele in isolated epithelial cells from *Pofut1* mutant intestine or colon (Fig S3A). As predicted, recombination was specific for small and large intestine; it was not detected in the stomach or other peripheral tissues (Fig S3B). *Pofut1*-deficient mice appeared normal at birth, but smaller than mice carrying either no or one recombined *Pofut1* floxed allele (Fig 1C). The mean reduction in weight was 42% in 4 week old *Pofut1*^{F/F}:villin-Cre mice compared to *Pofut1*^{F/F} littermates (n = 15) and this was associated with a modest decrease in survival (Fig S4).

Pofut1 has been shown in both *Drosophila* 16, 19 and mouse 18, to act upstream in the canonical Notch signaling pathway as a necessary component for the binding activity of Notch receptors to their ligands. In our model, functional blockage of Notch receptors signaling was achieved through inactivation of the *Pofut1* gene, as illustrated by dramatically reduced NICD1, NICD2 and HES1 expression (Fig 1D, Table S1) in intestinal and colonic epithelial cells of *Pofut1^{F/F}*:villin-Cre mice. *Hes5* transcripts were repressed specifically in the small intestine (in contrast to the colon) upon *Pofut1* gene excision. Concomitantly, *Math1* mRNA expression was enhanced (Table S1) and MATH1-positive cells accumulated in the crypts of the small intestine (Fig 1E), providing direct evidence that *Pofut1* expression is necessary for intestinal Notch signaling.

Secretory lineage production is increased in mouse intestine lacking *Pofut1*

The substantial elevation of all intestinal secretory cell lineages following *Pofut1* loss (Fig 2, Table S2) was reminiscent of *Hes1* or *Rbp-Jk* deletion 8, 9, providing strong evidence that POFUT1 targets Notch signaling in early crypt progenitors to control their lineage determination. Phenotypic alterations occurred mainly in the crypts and were homogeneous throughout the intestinal and colonic mucosa, again demonstrating highly efficient excision of the *Pofut1*-floxed alleles by the Cre transgene and the absence of a mosaic phenotype.

H&E staining (Fig 2A–B) and enhanced expression of lysozyme, *MMP7* and *Pla2g2a* (Fig S5A–B, S6A) showed that the Paneth cell population was greatly expanded in the small intestine of *Pofut1*-deficient animals (Fig S5E). The expression of SOX9, necessary for proper differentiation of this cell type 23, 24, was also upregulated (Fig S6D). Moreover, *Pofut1^{F/F}*:Villin-Cre mice exhibited goblet cell hypertrophy and hyperplasia in both the small and large intestine, as identified by Alcian blue and TFF3 staining (Fig 2C–D, S7, S5C–D), and by enhanced levels of *Tff3*, *Muc2*, *Fizz2*, and *Spdef* transcripts (Fig S6B). This expended goblet cell population secreted both neutral and acidic sialo- and sulfo-mucins (Fig S8). Most of these post-mitotic goblet cells accumulated in the crypt compartment, above the Paneth cells, where progenitors normally undergo rapid division (Fig 2D). Finally, the *Pofut1*-deficient intestine had an increased number of enteroendocrine cells all along the crypt-villus axis, although these cells resided more frequently in the vicinity of the crypt (Fig 2E–F, S5F). Moreover, the expression level of genes encoding gut hormones (*CCK*, *GIP*, *Glucagon*, *Ghrelin*, *Somatostatin*) revealed that all endocrine subtypes examined were expanded across the small intestine (Fig S6C, Table S2). Analysis of several transcription factors important for enteroendocrine specification and differentiation (*Insm1*, *NeuroD*, *Ngn3*, *Gfi1*) showed significantly increased expression in *Pofut1^{F/F}*:Villin-Cre small intestine (Fig S6D).

Pofut1 inactivation also enhanced secretory cell lineage differentiation at the modest expense of absorptive cell differentiation in the small intestine. In fact, attenuation in villus length (8%) (Fig S9A), and decreased transcript levels of several enterocyte specific genes (*Fabp2*, *Dpp4*, *lactase*, *Cdx2*; Fig S9B) suggested that the number of enterocytes was reduced in *Pofut1*-deficient intestine, although alkaline phosphatase staining and ultrastructural examination showed maintenance of the enterocyte brush border in *Pofut1^{F/F}*:Villin-Cre mice (Fig S9C–F). These data demonstrated that *Pofut1* loss triggers the binary choice of proliferating progenitors between differentiation toward secretory or absorptive cell lineages.

Altered proliferation of crypt progenitor cells in the absence of *Pofut1*

Loss of function experiments have shown that neither NOTCH1 nor HES1 are essential for crypt progenitor cell proliferation 9, 10, suggesting redundancy in Notch receptors 11 or Notch targets in stem cell maintenance, in the crypt epithelium. This is consistent with the

altered proliferation observed in crypt progenitors from *Pofut1*-deficient intestine, where the entire Notch signaling pathway is presumably turned off. Importantly, reduction in Ki67 staining was more pronounced in the colon of *Pofut1*-deficient mice compared to the small intestinal mucosa (Fig 3A–E). In parallel, the expression of the Cdk inhibitor p21 was upregulated in *Pofut1*-deficient intestine or colon whereas cyclin D1 protein level was decreased (Fig 3F). In the small intestinal epithelium, the transient amplifying compartment, which normally occupies the entire crypt (Fig 3A), was relocated to the upper part of the crypt, above the expanded secretory cell population (Fig 3B). Cell proliferation counts revealed a 25% reduction in proliferation, but paradoxically, there was an increase in total number of cells per crypt (Fig 3E) as well as a 1.6 fold increase in crypt depth (Fig S9A), likely reflecting the accumulation of secretory cells in the crypt of *Pofut1*^{F/F}:Villin-Cre mice. The slight reduction in migration of BrdU-labeled cells in the small intestine (Fig S10) suggested that secretory progeny migrated out of the crypts more slowly in *Pofut1*-deficient intestine. Alternatively, fewer cells may divide, but with a shortened cell cycle, producing a larger number of secretory cells. In the distal colon, proliferation of crypt progenitors was dramatically decreased (estimated as 65% of Ki67 positive cells per crypt) (Fig 3E). We did not observe intestinal or colonic crypt cells stained for cleaved caspase 3 or TUNEL in *Pofut1*-deficient mice (data not shown). Confirming a marked reduction in apoptosis in the *Pofut1*^{F/F}:Villin-Cre mice, both cleaved caspase 3 and PARP were undetectable in the colonic mucosa of these mice, in contrast to their prominence in the control *Pofut1*^{F/F} mice (Fig 3F).

Inactivation of Pofut1 leads to intestinal inflammation

The essential role of POFUT1 in maintenance of adult intestinal homeostasis was demonstrated by the chronic and diffuse entero-colitis that developed in 100% (18/18) of 36 week old *Pofut1*^{F/F}:Villin-cre mice. The inflammation was evident at 4 weeks, as shown by the thickened intestinal wall (Fig S7B, S7D) and the increased paracellular mucosal permeability (Fig S11), but severity of inflammation substantially increased in 36 week old *Pofut1*-deficient mice, in which changes were more pronounced in the colon (Fig 4A–D). The goblet cell hypertrophy and hyperplasia described above persisted in these older mice (Table I). The entero-colitis was characterized primarily by crypt hyperplasia, edema in the lamina propria with mixed inflammatory infiltrates, and frequent crypt abscesses and transmural inflammation. The colonic mucosa was thickened predominantly by T lymphocytes and macrophages, as demonstrated by CD3 and Mac-387 (Fig 4E–F) staining. This was consistent with enhanced production of chemoattractants for activated T cells and macrophages in total colonic mucosa from *Pofut1*^{F/F}:Villin-cre mice (Fig 5, Table S3). Increased expression of the $\alpha 1$, $\alpha 4$, $\beta 1$, and $\beta 2$ integrins and their ligands (MadCAM1, VCAM1, ICAM1 and fibronectin) likely contributed to immune cell adhesion to endothelium, mucosal homing, and activation of lymphocytes (Fig 5, Table S3). In addition, colonic secretions of the Th1 (IL2 and IFN γ), and the Th17 cytokines (IL17, IL23) were elevated after *Pofut1* deletion, whereas Th2 cytokines (IL4, IL10) production was not changed (Fig 5, Table S4). Macroscopically, the entero-colitis was accompanied by lymph vessel dilation (Fig 4G), a more prominent gut-associated lymphoid tissue (GALT) and occasional spleen hypertrophy (Fig 4H), suggesting a chronic immune stimulation.

Histopathologic examination of the small intestine of *Pofut1* mutant mice also demonstrated the presence of dysplastic foci (Fig 6A–B), morphologically distinct from aberrant crypt foci. These small nodules of dysplastic glandular epithelium had few to no goblet cells, no villi (i.e. were sessile lesions), and atypical epithelial cells, usually smaller than their adjacent non-dysplastic counterparts.

Gut microbiota population is altered in *Pofut1* deficient intestine

Although the cause of the inflammation was uncertain, we hypothesized that the enhanced production of mucus in *Pofut1*-deficient intestine and colon (Fig 6C, 6E) could result in changes in the mucus-associated flora, thereby allowing surface associated bacteria with increased inflammatory potential to become permanently established and contribute to the development of chronic inflammation. In fact, frequent crypt necrosis was observed in the intestinal and colonic mucosa of *Pofut1*^{F/F}: Villin-cre mice at 9 months (Fig 6D), suggesting bacterial infection.

To investigate this, we performed bacterial culture and histological scoring of Warthin-Starry stained (silver stain for spirilliform bacteria) sections from colonic mucosa of *Pofut1*^{F/F} and *Pofut1*^{F/F}:Villin-cre mice. Despite no significant overgrowth of aerobic or anaerobic bacteria associated with the colonic mucosa of the mutant mice, Gram-negative bacteria colonies were more frequently associated with the large intestinal mucosa in *Pofut1*^{F/F}:Villin-cre mice (Table II) and spiral-shaped organisms (interpreted to be *Helicobacter* spp) accumulated within the dilated and mucous-filled glands of the large intestine of *Pofut1*^{F/F}:Villin-cre mice (Fig 6F–G). Interestingly, Gram-negative bacteria translocation across the intestinal epithelium to mesenteric lymph nodes was observed exclusively in *Pofut1*^{F/F}:Villin-cre mice (Table II). In sum, these data suggest that disruption in the luminal environment of *Pofut1*-deficient mice contributes to an alteration in the composition of bacterial flora associated with the colonic mucosa which likely initiates the pronounced inflammation.

Discussion

Here, we report that blocking of all Notch/ligand interaction by targeted inactivation of the *Pofut1* gene in mouse small intestinal and colonic epithelia enhanced the commitment of crypt progenitor cells towards secretory cell lineages, and leads to hypersecretion of mucus, modification of the gut microbiota and the development of intestinal inflammation.

Consistent with previous studies 25, 26, we report that activation of Notch signaling occurs through concentration of Notch ligands and receptors predominantly in epithelial cells of the crypts, where *Pofut1* expression is the highest. The efficient down-regulation of NICD and HES1 expression, and the substantial elevation of all intestinal secretory cell lineages following *Pofut1* loss, which was reminiscent of *Hes1* or *Rbp-Jk* deletion 8, 9, provided strong evidence that POFUT1 targets Notch signaling in early crypt progenitors to control their lineage determination. Importantly, we show that POFUT1 is necessary for the transcriptional program of early secretory precursors, since expression of *Gfi1*, that selects Paneth/goblet versus enteroendocrine cell fates 27, is induced in *Pofut1*-deficient intestine. We also demonstrate that *Pofut1* loss controls an earlier event in proliferating progenitors where it triggers the binary choice between differentiation toward secretory or absorptive cell lineages, since reduced enterocytic differentiation was accompanied by an increased number of secretory cells. This is in accordance with previous *in vivo* experiments, in which inactivation of *Rbp-Jk* or *Hes1* led to a reduced number of differentiated enterocytes 8, 9, whereas only enterocytes emerged from *Math1*-mutant crypts 13.

The extent of phenotypic changes, such as proliferation index, observed in small compared to large intestine lacking *Pofut1* cannot be accounted for by differential expression of the O-fucosyltransferase. Rather, the differences likely arise from the complex expression pattern of Notch receptors and their ligands in the intestinal epithelium, which we have documented along the cephalo-caudal axis (Fig S1, S2). In fact, the type and the combination of the Notch ligand/receptor interactions in a given environment may have different effects on target gene expression 14, transcriptional activity of RBP-Jk and thus biological effect.

Interestingly, compared to the total absence of proliferating progenitors in *Rbp-Jk*-deficient intestine⁸, the more modest reduction in cell proliferation in *Pofut1*^{F/F}:Villin-cre mice was consistent with their viability (Fig S4). This discrepancy could underlie transcriptional activity of RBP-Jκ independent of NICD, as reported in the adult exocrine pancreas²⁸ and in 293T kidney cells²⁹. However, intestinal phenotypic changes in mice treated with a GSI were indistinguishable from those observed after *Rbp-Jk* deletion⁸, suggesting that Notch receptors might partially transduce signals independently of POFUT1 in the intestinal mucosa³⁰. An additional explanation for the differences between *Pofut1*- and *Rbp-Jk*-deficient mice might be a different expression pattern of POFUT1 and RBP-Jκ in crypt progenitors or stem cells. RBP-Jκ might target earlier intestinal progenitors than *Pofut1*.

The ability of *Pofut1* inactivation in the intestinal epithelium to induce both mucus hypersecretion and colitis was unexpected since colonic inflammation is usually accompanied by a compromised mucus barrier and goblet cell hypoplasia³¹. However, subsequent to the enhanced production of goblet cells in *Pofut1*-deficient mice, increased number of Gram negative bacteria associated with the mucus layer may contribute to the pronounced chronic inflammatory reaction, similar to abnormalities observed in the small intestine of the mouse model for cystic fibrosis³². Interestingly, the enhanced exposure of the colonic mucosa to spiral-shaped bacteria (such as *Helicobacter species*), which naturally colonize the mucus layer and accumulate in the crypts of *Pofut1*^{F/F}:Villin-cre, may trigger the strong Th1/Th17-associated adaptative immune response and the GALT hypertrophy. Infection with *Helicobacter spp.* has been associated with the development of T-cell mediated inflammatory bowel disease in various mouse models³³. Additional mechanisms independent of Notch pathway may be important. Glycosylation changes in gastro-intestinal POFUT1 target, such as Muc13³⁴, may affect bacterial adherence to the mucosa and then lead to increased inflammation. Moreover, the inflammation may be a predisposing factor for dysplastic lesions to progress to tumors in the *Pofut1*^{F/F}:Villin-cre mice, since 100% of the mice displayed mucosal inflammation and frequent dysplastic foci, though only one of ten *Pofut1*-deficient mice examined displayed an early stage adenoma in the small intestine (Fig S12). Thus, the *Pofut1*-deficient mouse may be a new model in which to dissect the complex relationship between colitis and elevated risk for colon cancer development and its modulation.

In conclusion, the reduced epithelial proliferation, and enhanced secretory cell differentiation in response to *Pofut1* inactivation demonstrate that the interactions between Notch receptors and ligands provide growth and differentiation signals in the intestinal proliferating cell compartment. Moreover, activation of Notch signaling, reflected by increased expression of HES1 and reduced HATH1 levels, has been described in human colorectal tumors and mouse models of intestinal tumorigenesis^{8, 12}. In this regard, down-regulation of Notch signaling through the use of γ -secretase inhibitors decreased cell proliferation and drove cells of Apc^{Min} mouse intestinal tumors to differentiate, and is thus a potential new approach for therapy of colon tumors⁸. However, we demonstrate that the overproduction of mucus following intestinal *Pofut1* deletion is paralleled by modifications of the gut microbiota that may contribute to the development of intestinal inflammation. Since alterations in lineage specific differentiation and mucus secretion are seen with loss of Notch signaling^{8, 11}, there is the potential that development of therapeutic approaches for colon tumors that target this pathway may have to address effects similar to those that develop longer term through *Pofut1* inactivation, including cytotoxicity of normal crypts, chronic intestinal inflammation, and dysplasia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Schweisguth F. Regulation of notch signaling activity. *Curr Biol*. 2004; 14:R129–38. [PubMed: 14986688]
- Fortini ME, Artavanis-Tsakonas S. The suppressor of hairless protein participates in notch receptor signaling. *Cell*. 1994; 79:273–82. [PubMed: 7954795]
- Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A. Signalling downstream of activated mammalian Notch. *Nature*. 1995; 377:355–8. [PubMed: 7566092]
- Zheng JL, Shou J, Guillemot F, Kageyama R, Gao WQ. Hes1 is a negative regulator of inner ear hair cell differentiation. *Development*. 2000; 127:4551–60. [PubMed: 11023859]
- Yang Q, Bermingham NA, Finegold MJ, Zoghbi HY. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science*. 2001; 294:2155–8. [PubMed: 11739954]
- Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S. Notch signals control the fate of immature progenitor cells in the intestine. *Nature*. 2005; 435:964–8. [PubMed: 15959516]
- Stanger BZ, Datar R, Murtaugh LC, Melton DA. Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A*. 2005; 102:12443–8. [PubMed: 16107537]
- van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*. 2005; 435:959–63. [PubMed: 15959515]
- Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD. Control of endodermal endocrine development by Hes-1. *Nat Genet*. 2000; 24:36–44. [PubMed: 10615124]
- Vooijs M, Ong CT, Hadland B, Huppert S, Liu Z, Korving J, van den Born M, Stappenbeck T, Wu Y, Clevers H, Kopan R. Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development*. 2007; 134:535–44. [PubMed: 17215306]
- Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimmer-Strobl U, Strobl LJ, Honjo T, Clevers H, Radtke F. Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27(Kip1) and p57(Kip2). *EMBO Rep*. 2008; 9:377–83. [PubMed: 18274550]
- Leow CC, Romero MS, Ross S, Polakis P, Gao WQ. Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. *Cancer Res*. 2004; 64:6050–7. [PubMed: 15342386]
- Shroyer NF, Helmrich MA, Wang VY, Antalffy B, Henning SJ, Zoghbi HY. Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. *Gastroenterology*. 2007; 132:2478–88. [PubMed: 17570220]
- Shimizu K, Chiba S, Saito T, Kumano K, Hamada Y, Hirai H. Functional diversity among Notch1, Notch2, and Notch3 receptors. *Biochem Biophys Res Commun*. 2002; 291:775–9. [PubMed: 11866432]
- Lei L, Xu A, Panin VM, Irvine KD. An O-fucose site in the ligand binding domain inhibits Notch activation. *Development*. 2003; 130:6411–21. [PubMed: 14627724]
- Okajima T, Irvine KD. Regulation of notch signaling by o-linked fucose. *Cell*. 2002; 111:893–904. [PubMed: 12526814]
- Okajima T, Xu A, Irvine KD. Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. *J Biol Chem*. 2003; 278:42340–5. [PubMed: 12909620]
- Shi S, Stanley P. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci U S A*. 2003; 100:5234–9. [PubMed: 12697902]

19. Sasamura T, Sasaki N, Miyashita F, Nakao S, Ishikawa HO, Ito M, Kitagawa M, Harigaya K, Spana E, Bilder D, Perrimon N, Matsuno K. neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development*. 2003; 130:4785–95. [PubMed: 12917292]
20. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem*. 2002; 277:33275–83. [PubMed: 12065599]
21. Mariadason JM, Nicholas C, L'Italien KE, Zhuang M, Smartt HJ, Heerdt BG, Yang W, Corner GA, Wilson AJ, Klampfer L, Arango D, Augenlicht LH. Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology*. 2005; 128:1081–8. [PubMed: 15825089]
22. Smartt HJ, Guilmeau S, Nasser SV, Nicholas C, Bancroft L, Simpson SA, Yeh N, Yang W, Mariadason JM, Koff A, Augenlicht LH. p27kip1 Regulates cdk2 activity in the proliferating zone of the mouse intestinal epithelium: potential role in neoplasia. *Gastroenterology*. 2007; 133:232–43. [PubMed: 17631145]
23. Mori-Akiyama Y, van den Born M, van Es JH, Hamilton SR, Adams HP, Zhang J, Clevers H, de Crombrughe B. SOX9 Is Required for the Differentiation of Paneth Cells in the Intestinal Epithelium. *Gastroenterology*. 2007; 133:539–46. [PubMed: 17681175]
24. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, Bibeau F, Scherer G, Joubert D, Hollande F, Blache P, Jay P. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol*. 2007; 178:635–648. [PubMed: 17698607]
25. Sander GR, Powell BC. Expression of notch receptors and ligands in the adult gut. *J Histochem Cytochem*. 2004; 52:509–16. [PubMed: 15034002]
26. Schroder N, Gossler A. Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene Expr Patterns*. 2002; 2:247–50. [PubMed: 12617809]
27. Shroyer NF, Wallis D, Venken KJ, Bellen HJ, Zoghbi HY. Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. *Genes Dev*. 2005; 19:2412–7. [PubMed: 16230531]
28. Beres TM, Masui T, Swift GH, Shi L, Henke RM, MacDonald RJ. PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Mol Cell Biol*. 2006; 26:117–30. [PubMed: 16354684]
29. Tang Z, Kadesch T. Identification of a novel activation domain in the Notch-responsive transcription factor CSL. *Nucleic Acids Res*. 2001; 29:2284–91. [PubMed: 11376147]
30. Stahl M, Uemura K, Ge C, Shi S, Tashima Y, Stanley P. Roles of pofut1 and o-fucose in Mammalian notch signaling. *J Biol Chem*. 2008; 283:13638–51. [PubMed: 18347015]
31. Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, Lochs H, Dorffel Y. Comparative study of the intestinal mucus barrier in normal and inflamed colon. *Gut*. 2007; 56:343–50. [PubMed: 16908512]
32. Norkina O, Burnett TG, De Lisle RC. Bacterial overgrowth in the cystic fibrosis transmembrane conductance regulator null mouse small intestine. *Infect Immun*. 2004; 72:6040–9. [PubMed: 15385508]
33. Zhang L, Mitchell H. The roles of mucus-associated bacteria in inflammatory bowel disease. *Drugs Today (Barc)*. 2006; 42:605–16. [PubMed: 17028670]
34. Rampal R, Luther KB, Haltiwanger RS. Notch signaling in normal and disease States: possible therapies related to glycosylation. *Curr Mol Med*. 2007; 7:427–45. [PubMed: 17584081]
35. Ito T, Udaka N, Yazawa T, Okudela K, Hayashi H, Sudo T, Guillemot F, Kageyama R, Kitamura H. Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. *Development*. 2000; 127:3913–21. [PubMed: 10952889]

FIGURE 1. Distribution and invalidation of *Pofut1* in mouse intestinal and colonic mucosa (A–B) Quantitative Real-Time PCR quantified the abundance of *Pofut1* mRNAs in (A) small or (B) large mouse intestine (denoted SI and LI, respectively). Epithelial cells were fractionated according to their proliferative/maturation status, fraction V (“villus”) including epithelial cells from the most apical part of the mucosa and fraction C (“crypt”) enriched in cells from the crypts. *Sox9* and *L-Fabp* expression were used to monitor tissue fractionation along the CVA. Results were expressed as mean $\pm$ SEM (n=5) and show relative mRNA expression after normalization to actin (arbitrary units). Values in fraction C were compared to fraction V by Student’s t-test (2 tailed, assuming equal variances; ns = non significant, ** = $p < 0.005$, *** = $p < 0.001$). (C) Mean growth curves (SEM bars are excluded for clarity) for *Pofut1*^{F/F}:Villin-cre mice (■), *Pofut1*^{F/+}:Villin-cre mice (▲), *Pofut1*^{+/+}:Villin-cre mice (x), and *Pofut1*^{F/F}:Villin-cre mice (□). (D) Total protein extracts from SI and LI epithelial cell lysates were separated by SDS-PAGE and analyzed by western blot for expression of NICD1 (antibody Val1744, Cell Signaling), NICD2 (antibody 100–401–408, Rockland), and the Notch targets HES1 (antibody kindly provided by Dr T. Sudo 35) and MATH1. β -actin was used as a loading control. (E) Representative sections of the small intestine (jejunum) from 4 week-old mice stained with MATH1 antibody (Chemicon). Scale bars: 10 μ m.

FIGURE 2. *Pofut1* deletion is associated with an expansion of secretory cell lineages in the intestinal epithelium

Representative sections of jejunum from 4 week-old *Pofut1*^{F/F} mice (left panels: A, C, E) and *Pofut1*^{F/F}:Villin-cre mice (right panels: B, D, F) stained for Paneth cells (H&E staining (A, B)), goblet cells (Alcian blue staining (C, D)), and endocrine cells (anti-Chromogranin A/B antibody (Abcam) (E, F)). Arrows indicate enteroendocrine cells (E) that accumulate in the crypts of *Pofut1*-deficient intestine. Scale bars: 50 μm and corresponding inserts 25 μm.

FIGURE 3. Abnormal proliferation in *Pofut1*^{F/F};Villin-cre mice

(A–D) Immunostaining for Ki67 (Vector Laboratories) labeled proliferating cells in small intestinal (SI) or large intestinal (LI) crypts from *Pofut1*^{F/F} control mice (white bars) and *Pofut1*^{F/F};Villin-cre mice (black bars). Scale bar: 50 μ m (A–D), 25 μ m (inserts). (E) Quantification of Ki67 positive cells (right), and total cell number (left) per crypt. White bars, crypts from *Pofut1*^{F/F}; black bars, *Pofut1*-mutant crypts. (F) Alteration of cell cycle regulators in epithelial cells from either small (SI) or large intestine (LI) from *Pofut1*^{F/F} control mice or their *Pofut1*^{F/F};Villin-cre littermates. Protein cell lysates were analyzed by Western blot to detect Cyclin D1 (Zymed Laboratories), p21 (Santa Cruz Biotechnology Inc.), cleaved caspase 3 and cleaved PARP expression (Cell Signaling Technology, Inc). β -actin was used as a loading control.

FIGURE 4. Inactivation of the *Pofut1* gene leads to intestinal and colonic inflammation

Representative sections of the jejunum (A, B) and the colon (C–F) from 36 week-old *Pofut1^{F/F}* mice and *Pofut1^{F/F} Villin-cre* mice. The colonic sections were stained for representation of (i) goblet cells by Alcian blue staining (A–D); (ii) lymphocytes by anti-CD3 antibody (Dako) (E); and (iii) macrophages by Mac-387 antibody (Santa Cruz Biotechnologies) (F). (D) Arrows indicate lymphoid nodules containing germinal centers, and the asterisk shows some cystic dilation of crypt with mucus. Scale bars: 50 μ m (A–B), 100 μ m (C–F). (G) Macroscopic image of the dissected colon from a 36 week old *Pofut1*-deficient intestine. The white arrow indicates a lymph vessel which has become obstructed. (H) Photograph of mouse spleen from 55 week-old *Pofut1^{F/F}* mice and *Pofut1^{F/F} Villin-cre* mice.

FIGURE 5. Cytokine profiling of whole colon culture supernates from *Pofut1*^{F/F}:Villin-cre mice Supernatants from 24h culture of *Pofut1*^{F/F}:Villin-cre mice (55 weeks) colon were analyzed with cytokine antibody array by using Mouse Cytokine Array (R&D Systems) according to the manufacturer’s instructions. Cytokine array membranes were incubated with 500 μ g total proteins at 4°C overnight. Results were expressed as mean $\pm$ SEM (n=4) and show optical density of each spot duplicate on the array. Values in *Pofut1*^{F/F} mice were compared to and *Pofut1*^{F/F}:Villin-cre mice by Student’s t-test (2 tailed, assuming equal variances; * = p<0.05, ** = p<0.005, *** = p<0.001).

FIGURE 6. Dysplastic foci, crypt abnormalities and associated spiral-shaped bacteria in *Pofut1^{F/F}*:Villin-cre mice

(A, B, D, E) H&E staining of jejunal sections from 36 week *Pofut1^{F/F}* and *Pofut1^{F/F}*:Villin-cre mice. (B) Arrows indicate sessile dysplastic foci. (D) Arrows indicate crypt necrosis, and (E) the asterisk shows crypt dilation with mucus. (C) Alcian blue staining of colonic section shows marked accumulation of mucus in 36 week old *Pofut1^{F/F}*:Villin-cre mice. (F–G) Warthin-Starry staining of colonic section from 36 week old mice showing increased spiral organisms in dilated crypts. Scale bars: 25 μ m (A, B, D, E), 10 μ m (F–G).

TABLE I

Histological scores of enterocolitis and helical bacteria content in Pofut1 deficient mice.

	Duodeno-jejunum		Colon	
Histological score (0–5) ^a	Pofut1^{F/F}	Pofut1^{F/F} Villin:cre	Pofut1^{F/F}	Pofut1^{F/F} Villin:cre
Crypt hyperplasia	0.0 ± 0.0	3.0 ± 0.0	0.5 ± 0.3	2.8 ± 0.2
Goblet cell hypertrophy	0.0 ± 0.0	2.0 ± 0.0	0.8 ± 0.2	2.8 ± 0.2
Goblet cell hyperplasia	0.0 ± 0.0	2.3 ± 0.2	0.5 ± 0.3	2.5 ± 0.3
Dilated crypts	0.0 ± 0.0	1.3 ± 0.2	0.5 ± 0.3	2.8 ± 0.2
Mixed inflammation	0.0 ± 0.0	1.3 ± 0.2	1.0 ± 0.0	2.3 ± 0.2
Lamina propria edema	0.0 ± 0.0	1.3 ± 0.2	0.0 ± 0.0	2.0 ± 0.0
GALT hyperplasia	0.3 ± 0.2	1.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
Dysplastic foci	0.0 ± 0.0	2.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Histological score	<i>Pofut1^{F/F}</i>	<i>Pofut1^{F/F} Villin:cre</i>	<i>Pofut1^{F/F}</i>	<i>Pofut1^{F/F} Villin:cre</i>
Warthin-starry positive ^b	nd	nd	+	++

^a data are expressed as mean±SEM of n=4 mice per genotype.

^b (+/-) equivocal helical bacteria in crypts (+) low or (++) high amount of helical bacteria in 1–5 crypts; (nd) not determined.

TABLE II

Bacterial translocation in mesenteric lymph nodes from *PofutI^{F/F}*; Villin-cre mice.

Group	Colonic mucosa	MLN
Mac Conkey II agar		
<i>PofutI^{F/F}</i>	$0.24 \cdot 10^{-3} \pm 0.11 \cdot 10^{-3}$	0
<i>PofutI^{F/F}</i> ; villin-cre	$39.90 \cdot 10^{-3} \pm 12.87 \cdot 10^{-3}$	$0.51 \cdot 10^{-3} \pm 0.37 \cdot 10^{-3}$
Trypticase Soy Agar With 5% Sheep's Blood		
<i>PofutI^{F/F}</i>	1.19 ± 0.55	0
<i>PofutI^{F/F}</i> ; villin-cre	1.60 ± 0.70	$6.71 \cdot 10^{-2} \pm 2.89 \cdot 10^{-2}$
CDC Anaerobe Blood Agar		
<i>PofutI^{F/F}</i>	36.37 ± 11.99	0
<i>PofutI^{F/F}</i> ; villin-cre	30.72 ± 11.35	2.45 ± 1.47

Values expressed as mean $\pm$ SEM (colony-forming units per microgram of tissue). Mac Conkey II agar, TSA, and CDC Anaerobes Blood agar colony counts were performed for 14 animals (7 mice/group). Mice were 55 week old. MLN indicates mesenteric lymph nodes.