Intestinal Deletion of *Pofut1* in the Mouse Inactivates Notch Signaling and Causes Enterocolitis

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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 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 of 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 enterocolitis. Therefore, these data identify important potential complications in strategies to target Notch signaling in therapeutic approaches to colon cancer.**

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 4 Notch genes (*Notch1* through *Notch4*) and 5 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-Jκ, which up-regulates the transcription of target genes,[2] including members of the hairy and enhancer of split (HES) and hairy related transcription factors. In particular, NICD stimulates expression of HES1,[3] 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 toward secretory cell types and continue to proliferate upon constitutive Notch1 activation.[6,7] Consistent with this, conditional inactivation of RBP-Jκ signaling drives cellular differentiation toward goblet and Paneth cell lineages in the mouse intestine.[8] Because Notch signaling seemed 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 principal that this pathway is a potential target for therapeutic approaches to colon cancer.[8]

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-Jκ, 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

Abbreviations used in this paper: BSA, bovine serum albumin; CVA, crypt-villus axis; GALT, gut-associated lymphoid tissue; GSI, γ-secretase inhibitor; HES, hairy and enhancer of split; HRP, horseradish peroxidase; NICD, Notch intracellular domain; MLN, mesenteric lymph nodes; PCR, polymerase chain reaction.

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interaction[14] 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 to signal through Delta-like and Jagged ligands.[15–19] 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 intestines 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$^{F/F}$ mice[18] 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 Jackson Laboratory (Bar Harbor, ME). Breeding generated mice that were homozygous for the *Pofut1* floxed allele and that harbored a Villin-Cre transgene. Mice were genotyped by polymerase chain reaction (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 intestines were removed immediately after killing 4- or 36-week-old mice, opened longitudinally, and inspected under a dissecting microscope.

Isolation and Fractionation of Epithelial Cells

The small and large intestines were dissected, everted, filled to distension with phosphate-buffered saline (PBS), and incubated with shaking at 37°C in 1.5 mmol/L 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 minutes, cell pellets were snap frozen in liquid nitrogen, and stored at −80°C.

Immunohistochemistry and Histologic 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% H_2O_2 for 10 minutes at room temperature and incubation of antibodies was performed overnight in bovine serum albumin (BSA)/PBS at 4°C. Antigen retrieval was achieved by boiling in 10 mmol/L citrate buffer pH 6.0 (30 minutes) for all antibodies, except anti-BrdU, which used 0.1% trypsin (20 minutes at 37°C). Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected with the diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA). For assay of alkaline phosphatase activity, the substrate (Vector red; Vector Laboratories) was applied to the sections for 10 minutes.

Histologic 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 mucus), and 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 radioimmunoprecipitation assay lysis buffer at 4°C, centrifugation at 15,000*g* for 15 minutes at 4°C, and supernatants were collected for Western blot analysis. Protein concentration was quantified using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Solubilized proteins (50–200 μg) were separated by electrophoresis on SDS-PAGE gels, transferred onto activated polyvinylidene difluoride membranes, and subjected to immunoblot analysis. Antibodies were then used according to the manufacturer's protocols. 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, Carlsbad, CA). First-strand cDNA was synthesized from 1 to 5 μg 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, Foster City, CA). 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