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## Glycans that regulate Notch signaling in the intestine

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### Abstract

Intestinal homeostasis is key to the maintenance of good health. The small intestine plays important roles in absorption, digestion, hormonal and immune functions. Crypt base columnar (CBC) stem cells residing at the bottom of crypts are nurtured by Paneth cells, and together create the stem cell niche, the foundation of intestinal homeostasis. CBC stem cells replicate to replenish their number, or differentiate into a variety of epithelial cells with specialized functions. Notch signaling is a cell-cell signaling pathway that regulates both the proliferation and differentiation of CBC stem cells. NOTCH1 and NOTCH2 stimulated by canonical Notch ligands DLL1 and DLL4 mediate Notch signaling in the intestine that, in concert with other signaling pathways including the WNT and BMP pathways, determines cell fates. Importantly, interactions between Notch receptors and canonical Notch ligands are regulated by O-glycans linked to Ser/Thr in epidermal growth factor-like (EGF) repeats of the Notch receptor extracellular domain (NECD). The O-glycans attached to NECD are key regulators of the strength of Notch signaling. Imbalances in Notch signaling result in altered cell fate decisions and may lead to cancer in the intestine. In this review, we summarize the impacts of mutations in Notch pathway members on intestinal development and homeostasis, with a focus on the glycosyltransferases that transfer O-glycans to EGF repeats of NOTCH1, NOTCH2, DLL1 and DLL4.

### Introduction

The intestine functions in the absorption and digestion of nutrients, and in endocrine and immune responses. The small intestine has renewal capability and is believed to be replaced almost entirely every 3–5 days in mice (1). Finger-like projections towards the lumen of the small intestine are called ‘villi’ and the invaginating regions in between are called ‘crypts’ (2). Intestinal epithelium consists of various cell types with unique functions. Multipotent stem cells expressing *Lgr5* and *Olfm4* reside at the bottom of crypts and are termed Crypt Base Columnar (CBC) stem cells (3). CBC stem cells self-renew or differentiate into several epithelial cell types generated via the absorptive lineage to produce enterocytes, or via the secretory lineage to produce antimicrobial peptide-secreting Paneth cells, hormone-secreting enteroendocrine cells, mucus-secreting goblet cells, and

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Tuft cells (4, 5). CBC stem cells initially give rise to intermediate progenitor cells which reside in the transit amplifying [TA] compartment until they differentiate into a specific epithelial cell type and migrate towards either the villus or the crypt. CBC stem cells also generate *Bmi1*-expressing cells at the +4 cell position from the most distal crypt cell (6). *Bmi1*<sup>+</sup> cells are the principle reserve stem cell that can generate *Lgr5*<sup>+</sup> CBC stem cells in response to stresses such as injury or inflammation (7–9). Notch signaling regulates the balance between *Bmi1*<sup>+</sup> and *Lgr5*<sup>+</sup> cell populations (7). However, cells of almost every differentiated type in the intestinal epithelium have been shown to function as reserve stem cells that give rise to *Lgr5*<sup>+</sup> CBC stem cells following stress (8, 9). In addition to Notch signaling, several other signaling pathways, including WNT, BMP, Hedgehog, EGF and mTORC1 regulate proliferation and differentiation of intestinal epithelium (10–14). These signaling pathways may function between intestinal epithelial cells of the crypt and villus themselves, and between mesenchymal cells, fibroblasts, telocytes or immune cells in surrounding tissue. Thus, the signaling niche supporting replication and differentiation of the intestinal epithelium is extremely complex (8), and poorly understood in both mouse and human. A recent spatio-temporal, single-cell RNA-seq study proposes cell-cell signaling complexities using a technique called Clump-seq (15), a beginning to unraveling functional pathways.

Notch signaling is a highly-conserved, cell-cell signaling pathway necessary for embryonic development and the maintenance of many tissues in the adult (16, 17). Defects in Notch signaling lead to developmental diseases, severe pathologies in adults, and initiation or progression of several cancers. Mammals have four Notch receptors (NOTCH1–NOTCH4) and five canonical Notch ligands (DLL1, DLL3, DLL4, JAG1, JAG2), all of which are single-pass, transmembrane proteins (18, 19). The extracellular domain of Notch receptors (NECD) contains 29–36 EGF repeats that carry O-glycans, followed by three cysteine-rich Lin12/Notch repeats (LNR), and two heterodimeric regions, which form the negative regulatory region. The Notch intracellular domain (NICD) contains an RBP-J $\kappa$ -associated module (RAM), and ankyrin (ANK) repeats, both needed for interactions with the transcriptional repressor RBP-J $\kappa$ . Nuclear localization signals (NLS) and a transcription transactivating domain (TAD) lie between the ANK repeats and a C-terminal PEST domain. Notch signaling is initiated by Notch ligands on neighboring cells binding to Notch receptors. The Notch/ligand interaction induces conformational changes in NECD that expose a site above the membrane for cleavage by ADAM10. (The related metalloprotease ADAM17 is not required for Notch signaling in mouse intestine (20)). Endocytosis of the released NECD into the ligand-expressing cell is followed by cleavage within the Notch transmembrane domain by  $\gamma$ -secretase. The released NICD goes to the nucleus where it forms a complex with CBF1-Suppressor of Hairless-LAG-1 (CSL, also known as RBP-J $\kappa$ ), Mastermind-like (MAML), and other transcription factors, to stimulate the transcription of many target genes (21). Several Notch target genes are transcriptional repressors, including the hairy and enhancer-of-split 1 (*Hes1*) family of basic helix-loop-helix transcription factors (22, 23).

## Notch signaling in intestinal epithelial cells

Each of the four Notch receptors and all five canonical Notch ligands are expressed in the small intestine (24, 25). At embryonic day E13.5, all five ligands and four Notch receptors are expressed in mesenchyme, while by E18.5 expression of ligands and Notch receptors has become scattered in different parts of intestine. At postnatal day P25, DLL1, DLL4, JAG1, JAG2, NOTCH1 and NOTCH2 are predominantly expressed within individual cells of the crypt while NOTCH3 and NOTCH4 are expressed in cells of the villus and the vasculature, respectively (24). In mouse intestinal organoids, JAG1, DLL1 and DLL4 are expressed in Paneth cells, DLL1 and DLL4 are expressed in goblet cells, while Notch receptors are activated in *Lgr5*<sup>+</sup> CBC stem cells (26). The glycosyltransferases that modify specific EGF repeats and regulate Notch signaling are also restricted in expression, as described below (26).

Cell fate decisions controlled by the Notch pathway in the intestine are summarized in Fig. 1. In the crypt, Notch receptors in CBC stem cells are activated by DLL1, DLL4 and JAG1 Notch ligands in Paneth cells. Absorptive or secretory progenitor cells in the TA region derive from CBC stem cells. Notch signaling stimulated by DLL1 in secretory lineage progenitors maintains the balance between absorptive and secretory progenitor cells by a Notch signaling mechanism termed lateral inhibition. The absorptive progenitors become enterocytes while secretory progenitors become goblet, enteroendocrine, Tuft or Paneth cells. Notch signaling strength is regulated by O-glycans attached to Ser/Thr in EGF repeats of the ECD of Notch receptors. Deletion of glycosyltransferase-encoding genes *Pofut1*, *Lfng* or *Rfng* has been shown to alter the balance of intestinal cell fates (7, 26, 27). Fig. 2 summarizes these findings and highlights outstanding questions concerning the interplay between different types of O-glycans on Notch receptors and Notch ligands. There are three classes of O-glycan named for the first sugar attached to an EGF repeat - O-fucose, O-GlcNAc and O-glucose. To date, only O-fucose glycans, initiated by the action of POFUT1 that transfers fucose from GDP-fucose in the endoplasmic reticulum (ER) to an EGF repeat, have been investigated in the intestine. The O-fucose on an EGF repeat becomes a substrate for Golgi enzymes Lunatic, Manic or Radical Fringe (LFNG, MFNG, RFNG), each of which transfers GlcNAc from UDP-GlcNAc to the O-fucose on an EGF repeat. The GlcNAc is often extended by the addition of galactose and sialic acid, by respective glycosyltransferases in the Golgi. O-glucose glycans and O-GlcNAc glycans are initiated in the same manner by their respective enzymes, POGLUT1 and EOGT (Fig. 2). Synergism between the three classes of O-glycans and their constituent sugars in regulating Notch signaling in the intestine is under investigation (28, 29).

Since Notch signaling is required for embryogenesis, conditional deletion must often be used to investigate roles for Notch signaling in intestine. The *VillCre*/1000 strain is highly specific for deletion in intestinal epithelium, although it may also delete in testicular germ cells (30). In this review, names of different mouse strains expressing *VillCre* and floxed allele(s) are the same as used in the original publication. Deletion of *Notch1* or *Notch2* by *VillCre*, compared to deletion of both together, revealed functional redundancy (31). Following tamoxifen injection, adult *Villin-CreERT2*;N1<sup>F/F</sup> mice exhibit downregulation of *Lgr5*, *Hes1* and *Bmi1*, and upregulation of *Atoh1/Math1*, leading to depletion of CBC stem

cells and increased production of Paneth, goblet, and enteroendocrine cells of the secretory lineage. The intestinal phenotype displayed by combined deletion of *Notch1* and *Notch2* in *Villin-CreERT<sup>2</sup>*; *N1<sup>F/F</sup>/N2<sup>F/F</sup>* mice is more severe, and essentially identical to the phenotype induced by treatment with  $\gamma$ -secretase inhibitors (32, 33) [Table 1]. Both *Notch1* and *Notch2* are required for crypt regeneration post-irradiation (34). Transgenic mice constitutively overexpressing NICD also exhibit altered cell fates in intestine (35, 36). Up-regulation of *Hes1* and concomitant downregulation of *Math1* and *Ngn3* transcripts were observed in mice with NICD overexpression, concomitantly with reduced differentiation of goblet and enteroendocrine cells, and increased CBC stem cell proliferation. Similarly, increased production of *Lgr5*-expressing stem cells was found in intestinal organoids overexpressing NICD (7).

Inducible deletion of *Dll1* in *Dll1<sup>flox/flox</sup>vil-Cre-ERT<sup>2</sup>* adult mice causes increased production of goblet cells in the intestine, but no changes in the expression of Paneth or enteroendocrine cells (37). Conditional deletion of *Dll4* or *Jag1* in intestine does not affect secretory lineage differentiation (37). Thus, removal of an individual ligand is not sufficient to recapitulate the altered cell fates observed when Notch signaling is inhibited by  $\gamma$ -secretase inhibitors, or in mice conditionally lacking both *Notch1* and *Notch2* (31–34). Inducible deletion of both *Dll1* and *Jag1* had the same effect on stem cell proliferation and differentiation as deletion of *Dll1* alone (37). Nevertheless, treatment of intestinal organoids with JAG1 induced the appearance of NICD, upregulation of *Hes1* and *Hes5* transcripts, and an increase in CBC stem cells, suggesting a Notch signaling role for JAG1 in intestine (7). Conditional deletion of both *Dll1* and *Dll4* causes massive formation of goblet cells and loss of *Lgr5*-, *Olfm4*- and *Acsl2*-expressing CBC stem cells (37). NICD1 was not detected in *Dll1-Dll4<sup>vil-Cre-ERT<sup>2</sup></sup>* intestine which exhibits a loss of *Hes1*-expressing cells, and elevated expression of *Math1* transcripts (37). Mice with conditional deletion of both *Notch1* and *Notch2* are viable for at least 12 days (31, 34), but *Dll1-Dll4<sup>vil-Cre-ERT<sup>2</sup></sup>* mice exhibit a rapid weight loss, cachexia, poor grooming and die 4–6 days after the last dose of tamoxifen (37). Taken together, these findings suggest that DLL1 is the main physiological ligand supporting Notch signaling in mouse intestine but, DLL4 is also necessary for optimal Notch signaling (37) [Table 1].

### O-Fucose glycans and Notch signaling in the intestine

Notch receptors and canonical Notch ligands are modified by O-glycans attached to most of the EGF repeats in their ECD (Fig. 2). The O-glycans regulate Notch ligand binding, and also may promote intracellular trafficking and stable expression at the cell surface (38–40). To investigate roles for O-fucose glycans in intestinal Notch signaling, the gene encoding protein O-fucosyltransferase 1 (*Pofut1*) was conditionally deleted using *Villin-Cre* (27). POFUT1 resides in the ER and transfers fucose from GDP-fucose to Ser (S) or Thr (T) in the consensus sequence C<sup>2</sup>XXXXS/TC<sup>3</sup> in an EGF repeat (41). *Pofut1* is ubiquitously expressed. Autosomal dominant mutations in *POFUT1* cause Dowling-Degos disease 2 (DDD2), characterized by reticulate hyperpigmentation (42). Mice lacking *Pofut1* die at ~E9.5 with severe developmental abnormalities typical of embryos lacking different members of the Notch signaling pathway (43, 44). X-ray crystals of NOTCH1 and DLL4 fragments in complex show that O-fucose on EGF12 of NOTCH1 interacts directly with

amino acids of DLL4 (45), and O-fucose on NOTCH1 EGF8 and EGF12 interact with the ligand binding domain of JAG1 (46). The biological importance of O-fucose on EGF12 was revealed in mice with a *Notch1* point mutation which precludes the addition of O-fucose to EGF12. Depending on genetic background, homozygous mutants exhibit defective T and B cell development (47), or die at ~E11.5 (48). Villin-Cre-mediated *Pofut1* conditional deletion (cKO) showed over-production of Paneth, goblet and enteroendocrine cells at the expense of absorptive enterocytes in intestinal epithelium, with accompanying crypt hyperplasia (27). The expression of *Hes1* was reduced, whereas *Math1* expression was increased. *Pofut1* cKO intestine exhibited T-lymphocyte infiltration, a Th1/Th17 immune response, bacterial translocation to mesenteric lymph nodes, altered species of mucus-associated gut microbiota, chronic intestinal inflammation, and formation of dysplastic foci, with occasional progression of tumors in small intestine as well as colon (27). Thus, lack of O-fucose transferred by POFUT1 leads to reduced Notch signaling, and altered cell fate decisions, as well as inflammation and adenoma in intestine. However, *Pofut1<sup>FF</sup>*:Villin-Cre mice do not die shortly after birth like those conditionally deleted for both *Dll1* and *Dll4* (37). This may be because other O-glycans on Notch receptors and ligands support Notch signaling in the absence of O-fucose glycans, as discussed below. *Lgr5*-EGFP-creER/*Pofut1<sup>flox/flox</sup>* mice were used to investigate whether Notch signaling regulates interconversion between *Bmi1<sup>+</sup>* reserve stem cells and *Lgr5<sup>+</sup>* CBC stem cells. A decreased ratio of *Bmi1<sup>+</sup>* to *Lgr5<sup>+</sup>* cells was observed following tamoxifen treatment (7). This correlated with loss of Notch signaling following *Pofut1* deletion. No NICD1 cleavage was seen in the intestine of *Lgr5*-EGFP-creER/*Pofut1<sup>flox/flox</sup>* mice, while expression of NICD1 via an *Lgr5*-EGFP-CreERT2/Rosa26-YFP-NICD transgene increased the *Bmi1<sup>+</sup>* to *Lgr5<sup>+</sup>* cells ratio [Table 2].

### Extension of O-fucose on Notch receptors

O-fucose on an EGF repeat provides a substrate for Fringe glycosyltransferases to transfer GlcNAc from UDP-GlcNAc for the generation of a GlcNAc $\beta$ 1,3Fuc O-glycan. This O-fucose glycan may be further extended by the addition of galactose and sialic acid, catalyzed by respective glycosyltransferases (49). Fringe was first reported as an essential gene required for wing formation in *Drosophila* (50), and subsequently shown to be a GlcNAc-transferase (51, 52). The three Fringe homologs LFNG, MFNG and RFNG, are expressed in intestine (24, 26). Mice globally deleted for *Mfng* do not exhibit significant changes in intestinal epithelium, but global deletion of *Lfng* or *Rfng* causes intestinal pathologies consistent with reduced Notch signaling (26).

Paneth cells in intestinal crypts support Notch signaling in adjacent CBC stem cells by expressing canonical Notch ligands (26, 53). Intestinal organoids in which *Rfng* is knocked down have fewer *Lgr5*-GFP<sup>+</sup> stem cells. Similarly, crypts from *Rfng* null mice are depleted for *Lgr5*-GFP<sup>+</sup> stem cells. Binding of NOTCH1-Fc was reduced on knockdown of *Rfng* in intestinal organoids, consistent with reduced DLL1 and DLL4 cell surface levels. Notch signaling and Notch targets HES1 and HEY1 were also reduced (26). Thus, *Rfng* promotes cell surface expression of DLL1 and DLL4 in Paneth cells, and enhances Notch signaling in CBC stem cells. Reduced cell surface expression of Notch ligands may reflect altered trafficking to, or stability at, the cell surface. A potentially direct, functional effect of

associated O-fucose glycans on canonical Notch ligands was not investigated. Evidence that O-fucose glycans on a Notch ligand may have a functional effect is from mutational analysis of *Dll3* to eliminate its O-fucose glycans (54). O-fucosylation-deficient *Dll3* could not fully rescue somitogenesis in mice lacking *Dll3* (54).

*Lfng* is most highly expressed by early progenitors of secretory cells in the transit amplifying (TA) zone in the upper crypt region, and in goblet, enteroendocrine and Tuft cells, throughout villi (26). Lineage tracing experiments show that *Dll1* is also expressed in the earliest progenitors of secretory cells. *Dll1*<sup>GFP</sup> progenitors derive from *Lgr5*<sup>+</sup> stem cells and may convert to *Lgr5*<sup>+</sup> stem cells in cultured organoids, and after irradiation of the intestine *in vivo* (55). *Lfng* knockdown in intestinal organoids leads to less cell surface expression of DLL1 and DLL4, and decreased NOTCH1-Fc binding to ligand-expressing goblet cells. Thus, LFNG also appears to play a supporting role in ligand expression at the surface, and to thereby promote Notch signaling in neighboring cells, especially in the lower crypt (26). *Lfng* knockdown in *Lgr5*-GFP<sup>+</sup> CBC stem cells reduces their colony-forming efficiency, suggesting that *Lfng* promotes Notch signaling in stem cells. Stem cells isolated from *Lfng* null mice exhibit low expression of *Hes1* and increased expression of *Math1* indicating reduced Notch signaling. *Lfng* null intestine exhibits accelerated secretory lineage differentiation with large numbers of Paneth, goblet and enteroendocrine cells (26) [Table 2]. Thus, in the intestine, both LFNG and RFNG act in signal-sending cells to promote cell surface expression of Notch ligands, and LFNG acts in CBC stem cells to promote Notch signaling and proliferation. Therefore, both Fringes function in regulating ligand-induced activation of Notch in CBC stem cells in the base of the crypt and also in Notch-controlled lateral inhibition in the TA zone, which maintains the balance of absorptive to secretory progenitors that give rise to differentiated cells of the villus. The high expression of *Lfng* in goblet, Tuft and enteroendocrine cells compared to CBC stem cells (26) is intriguing, and in need of further functional investigations.

### O-GlcNAc glycans of Notch receptors

O-GlcNAc on NOTCH was first discovered on EGF20 of *Drosophila* Notch (56). Subsequent studies identified an ER-resident, EGF-domain-specific glycosyltransferase (EOGT) that catalyzes the transfer of GlcNAc from UDP-GlcNAc to EGF repeats in the consensus site C<sup>5</sup>XXXX(T/S)GXXC<sup>6</sup> (57, 58). Mammalian O-GlcNAc can be further elongated by galactose and sialic acid in mammals (59). Defects in EOGT cause Adams-Oliver Syndrome 4 (AOS4), a rare, autosomal recessive disease (60). *Eogt* null mice are healthy and fertile (61). However, Notch signaling is impaired during post-natal retinal angiogenesis (61). *Eogt* deficiency decreases binding of Delta but not Jagged Notch ligands to certain cell types (61).

Roles for EOGT in intestinal epithelium have not been reported to date. In recent studies we have found that global deletion of *Eogt* caused only minor changes in morphology and gene expression in small intestine (28, 29). However, experiments to investigate whether O-GlcNAc glycans and EOGT might promote residual Notch signaling in the absence of POFUT1 revealed that both EOGT and POFUT1 contributed to Notch signaling. Global deletion of *Eogt* in combination with *Pofut1*<sup>F/F</sup>; Villin-Cre led to much more severe Notch



signaling defects than previously observed in *Pofut1<sup>F/F</sup>*:Villin-Cre mice (29). Ongoing studies are investigating differential roles for canonical Notch ligands in interacting with the altered Notch receptors in mice lacking both *Eogt* and intestinal *Pofut1*. In other experiments, mice lacking all Fringe activities in intestinal epithelium, either via global knockout or conditionally in *Lfng<sup>F/F</sup>Mfng<sup>-/-</sup>Rfng<sup>-/-</sup>*:Villin-Cre mice exhibited a defective Notch signaling phenotype, albeit with some features different from the *Pofut1*:Villin-Cre phenotype. Surprisingly, deletion of *Eogt* along with *Mfng* and *Rfng* globally and *Lfng* conditionally in *Eogt<sup>-/-</sup>Lfng<sup>F/F</sup>Mfng<sup>-/-</sup>Rfng<sup>-/-</sup>*:Villin-Cre mice had the effect of rescuing many aspects of the Notch-defective phenotype observed in triple *Fng* knockout intestinal epithelium (28). Roles for Notch ligands and Notch target genes in facilitating this rescue effect are under investigation.

### O-glucose glycans of Notch receptors

Roles for the O-glucose glycans (Fig. 2) in Notch signaling in intestine have not been reported. O-glucose is transferred to EGF repeats in the ER by POGLUT1 at Ser in the C<sup>1</sup>XSP/AC<sup>2</sup> consensus site, and the glucose may be extended by the addition of two xylose residues (62). Mutations in POGLUT1 cause Dowling Degos Disease 4 (DDD4) or Limb girdle muscular dystrophy R21 (LGMDR21) in humans (60). Cells lacking *Poglut1* have reduced Notch signaling but are not defective in Notch ligand binding. Deletion of *Poglut1* in the mouse is embryonic lethal (63).

### Notch signaling effectors downstream of Notch ligand binding

*Villin-CreERT2;Adam10<sup>f/f</sup>* mice (20) lose body weight rapidly and die 7–9 days after the first dose of tamoxifen. Both P0 null and conditional mutants exhibit altered intestinal morphology with reduced cell proliferation, increased apoptosis and increased numbers of Paneth, goblet and enteroendocrine cells at the expense of enterocytes. Expression of Notch targets *Hes1* and *HeyL* is reduced, while expression of *Math1*, *Ngn3*, *Spdef*, *Gfi1* and *Sox9* is upregulated. Similarly, organoids generated from *Adam10* cKO mice exhibit decreased cell proliferation and a reduction in stem cells (20).

Several investigations of the effects of  $\gamma$ -secretase inhibitor dibenzazepine (DBZ) revealed conversion of proliferative cells in intestinal crypts into goblet, Paneth and enteroendocrine cells (33, 64, 65). Treatment of mice or intestinal organoids by another  $\gamma$ -secretase small molecule inhibitor DAPT also leads to increased production of secretory pathway cells and a decreased ratio of *Bmi1<sup>+</sup>* to *Lgr5<sup>+</sup>* CBC stem cells (7, 33). Notch target genes are also dysregulated [Table 1]. Due to the well-established effects of  $\gamma$ -secretase inhibitors on Notch signaling, they are routinely used as a positive control to compare with results of other Notch pathway member deletions.

Conditional deletion of *Rbp-J $\kappa$*  leads to downregulation of *Hes1* and *Hes6* and upregulation of *Math1*, complete replacement of TA zone cells into post-mitotic goblet cells, and increased numbers of Paneth and enteroendocrine cells, indicative of enhanced differentiation to secretory cells (31, 32, 37, 64). Loss of Notch signaling upon *Rbp-J $\kappa$*  deletion results in loss of proliferative Ki67<sup>+</sup> and *Lgr5*-, *Olfm4*-, *Ascl2*-expressing CBC stem cells (32, 37). The intestinal phenotype of *Rbp-J $\kappa$*  cKO mice is similar to that

of *Notch1/Notch2* and *Dll1/Dll4* double cKO mice (31, 32, 34, 37). Intestine-specific expression of dominant-negative *Maml* (*dn-Maml*) also reduces *Hes1* expression and cell proliferation, and causes large increases in Paneth, goblet and enteroendocrine cells in newborn pups (20) [Table 1].

Downstream effectors of Notch signaling are members of the hairy and enhancer-of-split family basic helix-loop-helix transcription factors including HES1, HES3, HES5, HES7 and related HEY proteins including HEY1, HEY2 and HEYL (22). Small intestine from *Hes1* null embryos at E18 displays increased expression of *Atoh1* and *Atoh5* transcripts, consistent with increased production of enteroendocrine and goblet cells in the duodenum at E19 (66). Similarly, newborn *Hes1* null pups show enhanced mRNA levels of Paneth cell markers cryptdins 1, 4 and 5, and a slight increase in mRNA of lysozyme, compared to controls (67). Conditional deletion of *Hes1* at P2.5 leads to increased production of goblet, enteroendocrine and cleaved caspase-3<sup>+</sup> cells, but these phenotypes disappear when mice reach ~2 months of age (23). Interestingly, gene expression of *Hes3* and *Hes5* are upregulated in the intestine of *Hes1* mutants at E18, possibly to compensate for the deficiency of *Hes1* (66). Consistent with this interpretation, *Villin-Cre;Hes1<sup>F/-</sup>Hes3<sup>-/-</sup>Hes5<sup>-/-</sup>* mice show a marked increase in the production of goblet, Paneth, and enteroendocrine cells, increased apoptosis and decreased Ki67<sup>+</sup> proliferating cells at 2 month, in contrast to mice lacking only *Hes1* (23). *Hes5* deletion in the intestine results in upregulation of E3 ligase Fbw7 $\beta$  which destabilizes NICD1, inhibits lateral inhibition and leads to an increased number of goblet cells (68). *Hes1* deletion in adult mice causes upregulation of *Math1* which leads to excessive production of Paneth & endocrine cells, and mucus hyperplasia with microbial dysbiosis and inflammation (69). *Math1* null pups die just after birth due to failure of the respiratory system and defects in many essential cell lineages (70, 71). Intestine of *Math1<sup>\beta-Gal/-</sup>* at E18.5 shows a lack of goblet cells and enteroendocrine cells, and also loss of cryptdins in small intestine (72). Single cell RNA-seq indicated that *Atoh1/Math1* regulates the process by which CBC stem cells give rise to secretory cells (73–75). Thus, *Atoh1<sup>F/F</sup>:Fabp-Cre* mice expressing the *Fabpl<sup>4X AT-132</sup>Cre* transgene (76) or  $\beta$ -naphthoflavone-induced *Math1<sup>LoxP/LoxP</sup>:AHC* mice expressing Cre attached to the *Cyp1a* promoter (AH) (77), show drastic losses of goblet cells, Paneth cells and enteroendocrine cells in small intestine. This phenotype was not altered by treatment with the  $\gamma$ -secretase inhibitor DBZ which causes a massive formation of all secretory cells and loss of Ki67-expressing cells (65, 78). *Atoh1/Math1* behaves as a tumor suppressor in colorectal cancer (79, 80). Hence, mice with *Math1* deleted in intestine exhibit increased tumor formation in both adenomatous polyposis coli (APC)<sup>min</sup> and azoxymethane mouse models (78, 80) [Table 1].

## Conclusions

Roles for many members of the Notch signaling pathway have been identified in small intestine. Importantly, different functions of the various members have been revealed by individual and combined gene deletions, and by overexpression studies. Interestingly, the O-glycans on specific EGF repeats, the focus of this review, are required on the ECD of Notch receptors and DLL ligands for optimal Notch signaling in intestine. Loss of O-fucose glycans in *Pofut1*:Villin-Cre mice primarily reduces Notch signaling in CBC stem cells, with



many effects on cell fate decisions, whereas loss of *Rfng* reduces cell surface expression of DLL ligands in Paneth cells, and thus the induction of Notch signaling. Loss of *Lfng* affects Notch signaling in CBC stem cells, and Notch ligand cell surface expression in ligand-expressing cells. *Pofut1* deletion leads to mucosal inflammation, over-production of mucus, and altered microbiota, culminating in the formation of dysplastic foci with occasional tumor formation (27). In fact, increased expression of *POFUT1* is a marker of tumor progression in colorectal cancer in humans (81, 82), and activation of NOTCH1 signaling appears to be a driver of colorectal cancer (83).  $\gamma$ -Secretase inhibitors that inhibit Notch signaling cause intestinal toxicity which may be avoided by using a new Notch inhibitor 6-alkynyl fucose (6AF), that prevents ligand-induced Notch activation of both NOTCH1 and NOTCH2 (84).

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## Abbreviations

<b>ADAM</b>	a disintegrin and metalloprotease
<b>ANK</b>	Ankyrin repeats
<b>AOS4</b>	Adams-Oliver; Syndrome 4
<b>Ascl2</b>	Achaete-scute homolog 2
<b>BMP</b>	Bone Morphogenetic Proteins
<b>C2 domain</b>	module at the N-terminus of Notch ligands
<b>CBC</b>	Crypt base columnar
<b>CSL</b>	CBF-1 suppressor of Hairless-LAG-1
<b>DBZ</b>	dibenzazepine
<b>DDD</b>	Dowling-Degos disease
<b>DLL</b>	Delta like
<b>DSL</b>	Delta-Serrate-LAG-2 domain
<b>EGF</b>	epidermal growth factor-like
<b>EE</b>	enteroendocrine
<b>EOGT</b>	EFG-domain-specific glycosyltransferase
<b>ER</b>	endoplasmic reticulum
<b>Gfi1</b>	Growth factor independence 1
<b>GFP</b>	Green fluorescent protein

<b>GXYLT</b>	glucoside xylosyltransferase
<b>Hes</b>	hairy and enhancer split
<b>IRES</b>	An internal ribosome entry site
<b>JAG1</b>	Jagged 1
<b>LFNG</b>	Lunatic fringe
<b>LGMD</b>	limb-girdle muscular dystrophy
<b>Lgr5</b>	Leucine-rich repeat-containing G-protein coupled receptor 5
<b>LNRs</b>	Lin12/Notch repeats
<b>MAML</b>	mastermind-like
<b>Math1</b>	Mouse atonal homolog 1
<b>MFNG</b>	Manic fringe
<b>mTORC1</b>	Mammalian target of rapamycin complex 1
<b>NECD</b>	Notch extracellular domain
<b>Neu5Gc</b>	N-Glycolylneuraminic acid
<b>Ng3</b>	Neurogenin 3
<b>NICD</b>	Notch intracellular domain
<b>NRR</b>	Negative regulatory region
<b>O-GlcNAc</b>	O-linked GlcNAc or O-linked $\beta$ -N-acetylglucosamine
<b>Olfm4</b>	Olfactomedin-4 precursor
<b>POFUT1</b>	Protein O-fucosyltransferase 1
<b>POGLUT1</b>	Protein O-glucosyltransferase
<b>RAM</b>	RBP-J $\kappa$ associated module
<b>RBP-J <math>\kappa</math></b>	Recombination signal binding protein for immunoglobulin kappa J region
<b>RFNG</b>	Radical fringe
<b>Ser</b>	Serine
<b>Sox9</b>	SRY-Box Transcription Factor 9
<b>Spdef</b>	SAM pointed domain-containing Ets transcription factor
<b>Thr</b>	Threonine

**6AF** 6-alkynyl fucose**References**

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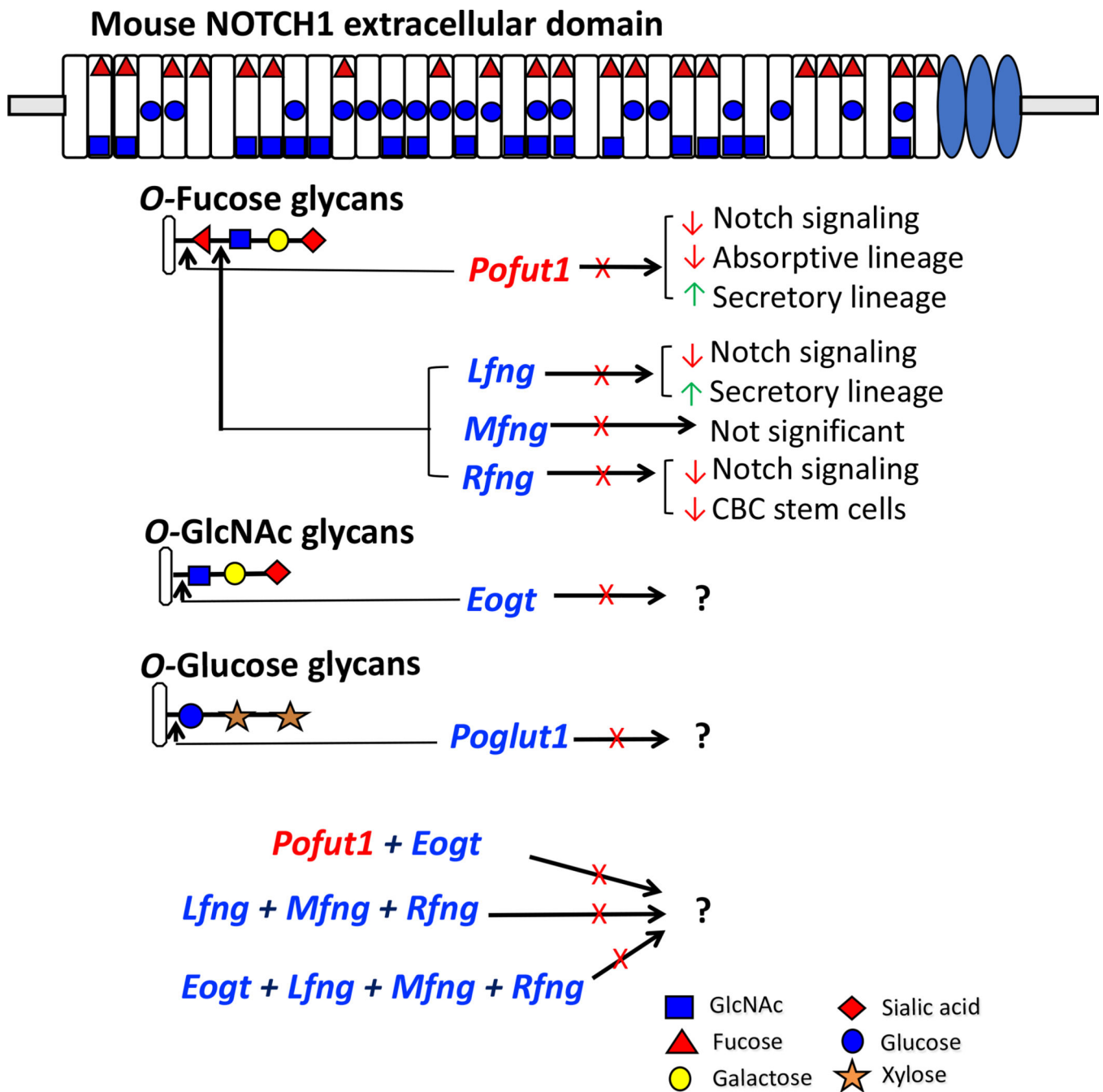


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### Perspectives

- Understanding the consequences of Notch signaling defects for intestinal development and maintenance, as well as roles for Notch signaling in colorectal cancer, are of critical importance to human health.
- Identifying different aspects of Notch signaling that regulate intestinal differentiation and homeostasis, and that are dysregulated in cancer, is a current focus. For example, loss of O-glycans transferred by POFUT1, LFNG or RFNG in the intestinal epithelium leads to different defects in Notch signaling, and provide insights into how the Notch signaling pathway is regulated in the intestine.
- Understanding synergies between different O-glycans in regulating Notch receptor-ligand interactions, their various effects on the strength of Notch signaling, and the consequences for cell fate decisions, will be important in identifying potential pathologies in human intestine arising from congenital or spontaneous mutations in the genes encoding Notch pathway members. Finding non-toxic inhibitors of Notch signaling to use in the treatment of colorectal cancer will also be important.





**Figure 2. Roles of O-glycans in Notch signaling in small intestine.**

Mouse NOTCH1 ECD consists of 36 EGF repeats which are modified by various O-glycans signified by the symbol for the initiating sugar. Each O-glycan is initiated at a consensus site in relevant EGF repeats by a specific glycosyltransferase as shown. The EGF repeats shown to be modified by a particular O-glycan are based on recent reviews for O-fucose and O-glucose (60) glycans, and for O-GlcNAc glycans (39). Further extension of the initial sugar by other glycosyltransferases leads to the formation of an O-glycan. The diagram summarizes the consequences of deleting different glycosyltransferase genes in

the intestine (X, gene deleted; red arrow, decrease; green arrow, increase). Proposed or ongoing experiments investigating intestinal consequences in compound mutant mice are also presented.

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Table 1

## Notch pathway mutant mouse models

Experimental model	Phenotype	Gene expression of Notch target genes		References
		Downregulation	Upregulation	
<i>Villin-CreERT2</i> ; N1 <sup>F/F</sup>	Increased goblet, enteroendocrine and Paneth cells. Decreased LGR5 <sup>+</sup> and EdU <sup>+</sup> cells. Loss of <i>Olfm4</i> <sup>+</sup> cells.	<i>Hes1</i> , <i>Lgr5</i> and <i>Olfm4</i>	<i>Math1</i> , <i>Ngn3</i> , <i>Spdef</i> , <i>Muc2</i> , <i>ChgA</i> , <i>Mmp7</i> , <i>Dll1</i> and <i>Dll4</i>	(34)
<i>Villin-CreERT2</i> ; N2 <sup>F/F</sup>	Unaffected goblet cells	Not reported	No changes in <i>Atoh1</i> , <i>ngn3</i> , <i>Muc2</i> , <i>ChgA</i> , <i>Mmp7</i> , <i>Dll1</i> and <i>Dll4</i>	(34)
<i>Notch1/Notch2</i> <sup>lox/lox</sup> - <i>vil-Cre-ERT2</i>	Highly increased goblet cells. Reduced <i>Hes1</i> <sup>+</sup> cells.	<i>Hes1</i>	<i>Muc2</i> , <i>Atoh1</i> , <i>Spdef</i> , <i>ChgA</i> , <i>Mmp7</i> , <i>Dll1</i> and <i>Dll4</i>	(31) (34)
<i>Villin-CreERT2</i> ; N1 <sup>F/F</sup> /N2 <sup>F/F</sup> (N1 <sup>-/-</sup> ;N2 <sup>-/-</sup> )				
Dll1 <sup>flx/flx</sup> <i>vil-Cre-ERT2</i>	Increased goblet cells	Not reported	Not reported	(37)
Dll4 <sup>flx/flx</sup> <i>vil-Cre-ERT2</i>	Unaffected goblet cells	Not reported	Not reported	(37)
Dll1-Dll4 <sup>vil-Cre-ERT2</sup>	Highly increased goblet cells. Decreased <i>Olfm4</i> <sup>+</sup> cells. Loss of <i>Lgr5</i> <sup>+</sup> , <i>Ascl2</i> <sup>+</sup> , <i>Hes1</i> <sup>+</sup> Ki67 <sup>+</sup> cells. Inhibited NICD1 cleavage.	Not reported	<i>Math1</i>	(37)
<i>Villin-Cre:Adam10</i> <sup>F/F</sup> <i>Villin-CreERT2:Adam10</i> <sup>F/F</sup>	Increased goblet, Paneth, enteroendocrine and cleaved caspase-3 <sup>+</sup> cells. Decreased enterocytes, BrdU <sup>+</sup> and Ki67 <sup>+</sup> cells.	<i>Hes1</i> , <i>HeyL</i> and <i>Olfm4</i>	<i>Tff3</i> , <i>Muc2</i> , <i>Cryptdins</i> , <i>Mmp7</i> , <i>ChgA</i> , <i>Atoh1</i> , <i>Gfi1</i> , <i>Spdef</i> , <i>Ngn3</i> and <i>Sox9</i>	(20)
$\gamma$ -secretase inhibitors DBZ/DAPT treatment to mice models or mouse intestinal organoid	Inhibited NICD1 expression. Increased goblet, Paneth enteroendocrine, Tuft, SOX9 <sup>+</sup> and MATH1 <sup>+</sup> cells. Decreased Ki67 <sup>+</sup> and HES1 <sup>+</sup> cells.	<i>Olfm4</i> and <i>Ascl2</i>	<i>Math1</i> , <i>Lysozyme</i> , <i>Mmp7</i> , <i>EphB3</i> , <i>Ngn3</i> , <i>Spdef</i> , <i>Gfi1</i> , <i>Tff3</i> and <i>Prom1</i> .	(7, 31–33, 64, 65, 78)
RBP-J <sup>flxed/flxed</sup> /P450-Cre <i>Rbpj</i> <sup>lox/lox</sup> - <i>vil-Cre-ERT2</i> RBP-J <sup>vil-Cre-ERT2</sup> <i>Rbpj</i> <sup>fl/fl</sup> ; <i>villin-creER</i> <i>Rbpj</i> <sup>F/F</sup> ; <i>Villin-CreERT2</i>	Increased goblet cells and MATH1 <sup>+</sup> cells. Reduced HES1 <sup>+</sup> cells. Loss of <i>Lgr5</i> <sup>+</sup> , <i>Olfm4</i> <sup>+</sup> , <i>Ascl2</i> <sup>+</sup> , <i>Hes6</i> <sup>+</sup> and Ki67 <sup>+</sup> cells.	<i>Hes1</i>	<i>Math1</i>	(32) (31) (37) (64) (34)
<i>Vil-Cre;Rosa26</i> <sup>DN-MAML/+</sup>	Increased goblet, Paneth and enteroendocrine cells. Decreased HES1 <sup>+</sup> and Ki67 <sup>+</sup> cells.	Not reported	Not reported	(20)
Embryonic <i>Hes1</i> KO <i>Hes1</i> -KO pups <i>Villin-Cre;Hes1</i> <sup>flx/-</sup> <i>Hes1</i> <sup>fl/fl</sup> Cre-ERT2 <i>Hes1</i> <sup>fl/fl</sup> <i>Villin-Cre</i>	Increased goblet, Paneth, cryptdin 4 <sup>+</sup> and cleaved caspase-3 <sup>+</sup> cells. Decreases Ki67 <sup>+</sup> cells.	<i>Reg3g</i> , <i>Saa1</i> and <i>Reg3b</i>	<i>Math1</i> , <i>Lysozyme</i> , <i>Cryptdin 1, 4 and 5</i> , <i>Gast</i> , <i>Gcg</i> , <i>Smst</i> , <i>Cck</i> , <i>Sct</i> , <i>Tph</i> , <i>Gip</i> , <i>ChgA</i> , <i>Atoh1</i> , <i>Isl1</i> , <i>Nkx2-2</i> , <i>Pax6</i> , <i>Pax4</i> , <i>Dll1</i> , <i>Dll3</i> , <i>Hes3</i> , <i>Hes5</i> , <i>Tbp</i> , <i>Atoh5</i> and <i>Neurod1</i> .	(66) (67) (23) (69) (69)
<i>Hes5</i> <sup>-/-</sup>	Increased goblet cells	Not reported	<i>Muc2</i> , <i>Dll1</i> and <i>Fbw7<math>\beta</math></i>	(68)
<i>Villin-Cre;Hes1</i> <sup>flx/-</sup> ; <i>Hes3</i> <sup>-/-</sup> ; <i>Hes5</i> <sup>-/-</sup>	Increased goblet, Paneth, enteroendocrine and cleaved caspase-3 <sup>+</sup> cell. Decreased Ki67 <sup>+</sup> cells.	Not reported	Not reported	(23)



Experimental model	Phenotype	Gene expression of Notch target genes		References
		Downregulation	Upregulation	
<i>Math1</i> <sup><math>\beta</math>-Gal/-</sup>	Decreased goblet, enteroendocrine and Paneth cells.	Lack of cryptdins	Not reported	(72)
<i>Math1</i> <sup>intestine/LacZ</sup>				(76)
<i>Math1</i> <sup>LoxP/LoxP/AHCre</sup>				(65)
<i>Atoh1</i> <sup>flx/flx;Fabp-Cre</sup>				(78)
<i>Atoh1</i> <sup>fl/fl;villin-cre<sup>ER</sup></sup>				(64)
<i>Lrig1</i> <sup>CreERT2/+;Atoh1<sup>fl/fl</sup></sup>				(75)

The name of each experimental mouse model is written as in the original article. The phenotype observed was based on histology, immunohistochemistry, Western analysis and flow cytometry. KO, knockout.

Table 2

## Glycosyltransferase mutant mouse models

Experimental model	Phenotype	Gene expression of Notch target genes		References
		Downregulation	Upregulation	
LGR5-EGFP-creER/ POFUT-1 <sup>flox/flox</sup> <i>Pofut1</i> <sup>F/F</sup> ; Villin-Cre	Reduced ratio of BMI1 <sup>+</sup> /LGR5 <sup>+</sup> cellsIncreased goblet, Paneth and enteroendocrine cells. Decreased HES1. Increased MATH1. Inhibited NICD1 and NICD2 cleavage.	<i>Hes1</i>	<i>Notch3, Dll4, Math1,</i> <i>Insm1, Neurod1,</i> <i>Neurog3, Gfi1</i> and markers of goblet, Paneth and enteroendocrine cells.	(7) (27)
<i>Lfng</i> null <i>Lfng</i> KD organoids	Increased goblet cells. Inhibited NICD1 cleavage. Reduced NOTCH1- Fc binding. Decreased DLL1 and DLL4 expression.	<i>Hes1</i>	<i>Atoh1</i>	(26)
<i>Rfng</i> null <i>Rfng</i> KD organoids	Depletion of CBCs. Reduced HES1 and HEY1. Reduced NOTCH1-Fc binding. Decreased DLL1 and DLL4 expression.	<i>Lgr5</i>	Not reported	(26)
<i>Mfng</i> null <i>Mfng</i> KD organoids	No significant phenotypes observed.	No change in <i>Lgr5,</i> <i>Notch1, Hes1, Hes5,</i> <i>Dll1, Dll4</i> and <i>Jag1.</i>	Not reported	(26)

The name of each experimental mouse model is written as in the original article. The phenotype observed was based on histology, immunohistochemistry, Western analysis and flow cytometry. KD, knockdown.