Regulation of myeloid and lymphoid cell development by O-glycans on Notch

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Notch signaling via NOTCH1 stimulated by Delta-like ligand 4 (DLL4) is required for the development of T cells in thymus, and NOTCH2 stimulated by Notch ligand DLL1 is required for the development of marginal zone (MZ) B cells in spleen. Notch signaling also regulates myeloid cell production in bone marrow and is an essential contributor to the generation of early hematopoietic stem cells (HSC). The differentiation program in each of these cellular contexts is optimized by the regulation of Notch signaling strength by O-glycans attached to epidermal growth factor-like (EGF) repeats in the extracellular domain of Notch receptors. There are three major types of O-glycan on NOTCH1 and NOTCH2 - O-fucose, O-glucose and O-GlcNAc. The initiating sugar of each O-glycan is added in the endoplasmic reticulum (ER) by glycosyltransferases POFUT1 (fucose), POGLUT1/2/3 (glucose) or EOGT (GlcNAc), respectively. Additional sugars are added in the Golgi compartment during passage through the secretory pathway to the plasma membrane. Of particular significance for Notch signaling is the addition of GlcNAc to O-fucose on an EGF repeat by the Fringe GlcNAc-transferases LFNG, MFNG or RFNG. Canonical Notch ligands (DLL1, DLL4, JAG1, JAG2) expressed in stromal cells bind to the extracellular domain of Notch receptors expressed in hematopoietic stem cells and myeloid and lymphoid progenitors to activate Notch signaling. Ligand-receptor binding is differentially regulated by the O-glycans on Notch. This review will summarize our understanding of the regulation of Notch signaling in myeloid and lymphoid cell development by specific O-glycans in mice with dysregulated expression of a particular glycosyltransferase and discuss how this may impact immune system development and malignancy in general, and in individuals with a congenital defect in the synthesis of the O-glycans attached to EGF repeats.

KEYWORDS
Notch signaling, O-fucose, O-GlcNAc, Fringe, HSCs, lymphoid, myeloid

Introduction

Notch signaling is a highly conserved pathway that plays a pivotal role in the maintenance of tissue development and homeostasis. Defective Notch signaling leads to numerous pathological conditions (Varshney and Stanley, 2018; Zhou et al., 2022). In mammals, there are four Notch receptors (NOTCH1-NOTCH4) and five ligands of the
Jagged and Delta-like families (DLL1, DLL3, DLL4 - orthologs to fly Delta; JAG1, JAG2 - orthologs to fly Serrate). Both Notch receptors and the canonical ligands, are transmembrane proteins with an extracellular domain (ECD) that consists primarily of epidermal growth factor-like (EGF) repeats (Figure 1). Many of these EGF repeats harbor consensus sites for the addition of different types of O-glycans to Ser or Thr, which takes place in the endoplasmic reticulum (ER) (Saiki et al., 2021). Classical Notch signaling is induced when Notch receptors interact with Notch ligands in neighboring cells (Kopan, 2012; Hori et al., 2013) (Figure 1). The binding of ligand promotes two proteolytic cleavages of a Notch receptor. The first is catalyzed by an ADAM-family metalloprotease and releases the extracellular domain, which is endocytosed into the ligand-expressing cell (Seib and Klein, 2021). The second cleavage is mediated by γ-secretase, an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1 whose action releases the Notch intracellular domain (ICD), which translocates to the nucleus and cooperates with the DNA-binding protein RBP-Jκ/CSL and a co-activator Mastermind-like 1 (MAML1), to promote transcription and activate Notch target genes (Kovall et al., 2017). There are numerous factors involved in the regulation of Notch signaling, including the expression of Notch ligands in signal-receiving cells that may cause cis-inhibition of Notch receptors, factors affecting secretory pathway trafficking, and the complement of O-glycans attached to the Notch ECD.

The major O-glycans found on EGF repeats of Notch receptors and the canonical Notch ligands are initiated by three different sugars: 1) O-fucose is added to Ser or Thr in the consensus site C2XXXX(S/T)C3 between the second and third Cys residues, where X is any amino acid, and either Ser or Thr accepts a fucose transferred from GDP-fucose by protein O-fucosyltransferase 1 (POFUT1); 2) O-glucose is added to Ser in the consensus site C 1XSX(P/A)C2 where Ser accepts glucose from UDP-Glc transferred by protein O-glucosyltransferase 1 (POGLUT1); and 3) O-GlcNAc added to Ser/Thr in the consensus site C5XXGX(S/T)GXXC6 where Ser or Thr accepts GlcNAc transferred from UDP-GlcNAc by EGF domain-specific O-linked N-acetylglucosaminyltransferase (EOGT) (Saiki et al., 2021) (Figures 2A,B). The consensus
sequence for POGlut2 and POGlut3 is distinct from that of POGlut1 (Takeuchi et al., 2018a) (Figure 2B). The four mammalian Notch receptors contain large numbers of EGF repeats in their respective ECD (Figure 2C), the Notch ligands contain fewer (Figure 2D). The predicted O-glycan occupation of NOTCH1 based on EGF consensus sequences is shown in Figure 2C. Almost all structural studies to determine O-glycan occupancy have been performed on NOTCH1 constructs expressed in cells overexpressing different O-glycan glycosyltransferases. To date there is no O-glycan analysis of a Notch receptor purified directly from a mammalian physiological source. However, species comparisons of O-glycan consensus sites in EGF repeats reveal that some are highly conserved like the O-fucose site in EGF12, and others may vary between species (Haines and Irvine, 2003; Saiki et al., 2021). Each of the initiating sugars can be extended by the addition of other sugars to form a glycan. O-fucose is extended by the transfer of GlcNAc from UDP-GlcNAc by one of three Fringe GlcNAc-transferases termed LFNG, MFNG and RFNG. The GlcNAc may be extended by Gal, which may in turn be extended by sialic acid (Sia) to generate the O-linked tetra-saccharide Sia-Gal-GlcNAc-Fuc-O-EGF. Structural analyses of NOTCH1 overexpressed in HEK293T cells showed that O-fucose consensus sites almost always have a fucose, but the degree of extension of each fucose is variable (Kakuda and Haltiwanger, 2017). In T cells from mouse spleen activated in culture, Fringe modification of NOTCH1 occurs at few O-fucose residues (Matsumoto et al., 2022). O-glucose may be extended by xylose transferred from UDP-Xyl by xylosyltransferases GXYLT1 or GXYLT2, and a second Xyl may be added by XXYLT1 (Yu and Takeuchi, 2019). O-GlcNAc may be extended by Gal and Sia as well as Fuc (Tsukamoto et al., 2022). Several congenital human diseases arise from mutations in genes encoding the glycosyltransferases that modify EGF repeats in Notch receptors and ligands (Varshney and Stanley, 2018; Matsumoto et al., 2021).
In this review, we summarize our current understanding of roles for O-glycans in Notch signaling during hematopoiesis, myeloid and lymphoid cell development in the mouse. We also discuss how different O-glycans may impact immune cell development and malignancy, in general, and in individuals with a congenital defect in the synthesis of the O-glycans attached to Notch receptors.

**Notch signaling in hematopoiesis**

The yolk sac is the first organ with hematopoietic potential in the mouse embryo, producing mainly nucleated red blood cells (RBCs) and a few macrophages and megakaryocytes during primitive hematopoiesis, beginning at embryonic day E7.5 (Pajcini et al., 2011; Bigas et al., 2012). Hematopoiesis continues in the embryo at sites of hemogenic endothelium, initially in the aorta-gonad-mesonephros (AGM), which forms HSCs that migrate to the fetal liver, and from there to the bone marrow (BM), to finally give rise to all erythroid, myeloid and lymphoid cells in the adult. Variations in the strength of Notch signaling are crucial for the switch between the formation of endothelial cells (low Notch signal induced by JAG1), and the formation of HSCs (high Notch signal induced by DLL4) from hemogenic endothelium (Gama-Norton et al., 2015; Porcheri et al., 2020). Abolishing Notch signaling has no effect on hematopoiesis in the yolk sac (Pajcini et al., 2011; Bigas et al., 2012). However, signaling via NOTCH1, but not NOTCH2, is required for the generation of HSCs in the embryo, although not for their proliferation or maintenance in the adult (Kumano et al., 2003; Soulhol et al., 2016). On the other hand, various experimental approaches that increase Notch signaling in HSCs lead to increased proliferation and expansion of the stem cell pool (Lampaia et al., 2017). Most recently, conditional deletion of RBP-Jk in HSC has shown that Notch signaling, while not required for the generation or maintenance of long- or short-term HSCs, is important for HSC self-renewal after irradiation or chemotherapeutic stress (Lakhan and Rathinam, 2021).

Roles for individual Notch receptors and ligands in hematopoiesis have been identified by conditional knockout strategies in the mouse. Overall requirements of Notch signaling have been investigated by inactivation of downstream transcription factors including RBP-Jk, that are required for signaling through all canonical Notch receptor/ligand interactions (Kovall et al., 2017). Inactivation of Notch1 in HSCs using Mxi1-Cre revealed that NOTCH1 is essential for the development of T cells in the thymus (Radtke et al., 1999; MacDonald et al., 2001). Conditional deletion of Notch1 in thymic T cells using a CD4-Cre revealed requirements for NOTCH1 in the generation of T cells involved in innate immunity in liver, intestine and spleen (Chennupati et al., 2016). A role for NOTCH2 in early T cell development was recently identified in co-culture investigations of Notch1 and Notch2 following inactivation in bone marrow progenitors (Romero-Wolf et al., 2020). In elegant rescue experiments of HSCs conditionally-deleted for RBP-Jk, the downstream effector of Notch signaling, a block in the development of thymus-seeking T cell progenitors in bone marrow was identified (Chen et al., 2019).

Experiments to determine roles for Notch ligands in thymic stromal cells used Foxn1-Cre to identify DLL4 as essential for stimulating Notch signaling in the thymus (Hozumi et al., 2008; Koch et al., 2008). However, recent rescue experiments have shown that transgenic Dll1 can substitute for Dll4 when Dll4 is deleted, and can stimulate NOTCH1 and NOTCH2 to promote T cell development in thymus (Hirano et al., 2022). Immunochemistry shows that DLL1, DLL4, JAG1 and JAG2 Notch ligands are expressed in thymus in a regio-specific manner which may be important in controlling Notch signaling strength in different regions of the thymus (Felli et al., 1999; Garcia-Leon et al., 2018).

Notch2 is expressed in Pro B and Pre B cells of the bone marrow, and in the different B cell subsets of spleen (Garis and Garrett-Sinha, 2021). However, conditional deletion of Notch2 using Mxi1-Cre primarily inhibits development of MZ B cells and their precursors in spleen (Saito et al., 2003). DLL1 in splenic fibroblasts is the Notch ligand responsible for stimulating Notch signaling via NOTCH2 to generate MZ B cells in spleen (Sheng et al., 2008). Plasticity in this differentiation has recently been revealed in the generation of MZ B cells from follicular B (Fo B) cells (Lechner et al., 2021). A diagram of bone marrow HSC differentiation into lymphoid and myeloid cells in thymus and spleen including expression of Notch receptor, Notch ligand and Notch-related glycosyltransferase genes from the ImmGen Project (Immunological Genome Project, 2020) is shown in Figure 3.

**Notch target genes in lymphopoiesis and myelopoiesis**

Determining genes altered in expression following deletion of Notch pathway members in HSCs or progenitors must be performed in the earliest lineage in which the gene is expressed because an essential requirement for Notch signaling may mean that certain cell populations are not generated or are contaminated by cells that differentiate along aberrant pathways. For example, deletion of Notch1 using Mxi1-Cre does not appear to perturb bone marrow cell populations derived from HSCs, but blocks T cell development at an early stage, and causes T cell progenitors to differentiate into B cells and myeloid cells in the thymus (Radtke et al., 1999; Feyerabend et al., 2009). Consequently, gene expression analysis of CD4/CD8 double-negative (DN) thymocytes lacking Notch1 which have become B cells and myeloid cells, would not provide insights into genes related to T cell development. Interestingly, deletion of
RBP-Jκ in HSCs using Vav1-Cre or Mx1-Cre does not affect Notch target gene expression in HSCs, and actually increases expression of classic Notch target genes like Hes1 and Hes5 in megakaryocyte, erythroid and myeloid bone marrow progenitors (Duarte et al., 2018). However, investigations of thymus-seeding progenitors in bone marrow by an elegant gene rescue approach revealed requirements for RBP-Jκ and Notch signaling in the generation of the MPP4/LMPP lymphoid progenitor population in bone marrow (Chen et al., 2019). Thus, Notch signaling is required for generation of thymus-seeding progenitors in bone marrow. In the case of Notch target Hes1, conditional knockout with Mx1-Cre does not affect HSC or HSPC numbers but causes a major block in T cell development in thymus, as expected if Notch signaling is inhibited (Wendorff et al., 2010). However, MZ B cell numbers in spleen are not altered following the loss of Hes1 in HSCs (Wendorff et al., 2010). Deletion of Hes1 using Vav1-Cre also showed that hematopoiesis was not obviously altered. However, stress hematopoiesis was ineffective in stem cells lacking Hes1, which exhibit an exhausted phenotype (Ma et al., 2020).

**Strategies to identify roles for O-glycans in hematopoiesis**

As mentioned above, Notch receptors and ligands are transmembrane glycoproteins with an ECD comprising numerous EGF repeats (Figures 2C,D). Each EGF repeat consists of ~40 amino acids, including six conserved cysteines which form three disulfide bonds. EGF repeats may contain consensus sequences for the addition of fucose (Fuc), glucose (Glc) or N-acetylglucosamine (GlcNAc) to Ser or Thr to initiate an O-Fuc, O-Glc or O-GlcNAc glycan (Haltiwanger et al., 2022). Glycosyltransferases that transfer an initiating sugar reside in the ER - POFUT1 transfers Fuc from GDP-Fuc, POGLUT1/2/3 transfer Glc from UDP-Glc, and EOGT transfers GlcNAc from UDP-GlcNAc. O-fucose on an EGF repeat may be extended by Fringe GlcNAc-transferases LFNG, MFNG or RFNG, and the GlcNAc may be further extended by Gal and sialic acid (Sia). O-glucose may be extended by xylose (Urata et al., 2020) and O-GlcNAc may be extended by Gal and Sia (Ogawa et al., 2020). To date, experiments aimed at defining roles...
for O-glycans on EGF repeats in immune cell development have been performed by manipulating the O-fucose glycans by deleting or overexpressing Pofut1, Lfng, Mfng or Rfng (Figure 2A). Only approximately 100 proteins contain EGF repeats that may be modified by these O-glycans (Rampal et al., 2007). The four Notch receptors and five Notch ligands comprise almost 10% of these substrates. However, ascribing phenotypic changes observed after deletion or overexpression of one of these glycosyltransferases to changes in Notch signaling depends on whether the phenotype mimics that of mutants deleted for, or overexpressing, other Notch pathway members; or on the mutation causing defective activation of Notch receptors by ligand-induced cleavage to generate NICD; or on the altered expression of known Notch signaling target genes. To directly determine if loss of one or more O-glycans on a particular Notch receptor or Notch ligand provides the basis of a glycosylation-defective phenotype, it is necessary to mutate individual Ser/Thr residues to preclude glycosylation in a specific EGF repeat. This, however, comes with caveats, since the alternative amino acid chosen can affect the outcome, as described below.

O-Fucose glycans and Notch signaling in lymphopoiesis and myelopoiesis

Deletion of Pofut1 in mice results in embryonic lethality with a phenotype typical of defective Notch signaling (Shi and Stanley, 2003; Okamura and Saga, 2008). Conditional deletion of Pofut1 in bone marrow HSCs using Mx1-Cre or Vav1-Cre causes changes in lymphoid and myeloid cell differentiation (Yao et al., 2011; Yu et al., 2015). HSC progenitors (Lineage minus (Lin-)Sca1+cKit + bone marrow cells termed LSK) lacking Pofut1 exhibit a 3-6-fold reduction in the binding of soluble Notch ligands DLL1-Fc and DLL4-Fc, a major decrease in ligand recognition, and a slight reduction of ~30% in cell surface expression of NOTCH1 and NOTCH2 (Yao et al., 2011), indicating a minor effect on Notch receptor trafficking. Myeloid cells in bone marrow were increased, whereas T cell production in thymus, and MZ B cells in spleen were decreased, as expected if the loss of O-fucose glycans causes defective Notch signaling via both NOTCH1 and NOTCH2. In addition, expression of Notch target genes Dtx1 and Hes1 was depressed in co-cultures of LSK cells with OP9 stromal cells under conditions that induce differentiation to T cells. Bone marrow transplantation revealed that the reduced lymphopoiesis and altered myelopoiesis following conditional deletion of Pofut1 arose largely from cell-autonomous effects in HSCs and HSC progenitors (HSPC). The overall findings validated conclusions of earlier experiments that characterized defective myelopoiesis in mice with a global knockout of Gmds, an enzyme required for the synthesis of GDP-Fuc, the substrate of POFUT1 (Zhou et al., 2008). Loss of O-fucose on Notch receptors, proposed as the basis of changes in myelopoiesis in the Gmds [--/--] mice, is consistent with the Notch-defective phenotype of mice lacking Pofut1 the enzyme that transfers O-fucose to all Notch receptors and ligands (Yan et al., 2010). Interestingly, conditional deletion of the downstream transcription factor RBP-Jκ that transduces Notch signaling via all combinations of Notch receptors and ligands, gives more severe reductions in lymphoid and myeloid cells than deletion of Pofut1 in HSC (Yu et al., 2015), suggesting that other O-glycans on Notch receptors might support Notch signaling in the absence of O-fucose glycans. We recently addressed this question by generating mice lacking Eogt and O-GlcNAc glycans as well as being conditionally deleted for Pofut1 in HSCs, as described below (Tanwar and Stanley, 2022).

An alternative approach to deleting Pofut1 is to pinpoint functions of O-fucose in NOTCH1 by mutating key O-fucose sites in EGF repeats of the ECD. The first example in mouse was mutation of the O-fucose site in EGFI2 of NOTCH1 from Thr to Ala (Ge and Stanley, 2008). EGFI2 is within the Notch ligand binding domain, and we now know that the O-fucose in EGFI2 of NOTCH1 is recognized directly by Notch ligands (Luca et al., 2015; Luca et al., 2017). Homozygotes with the EGFI2 mutation termed Notch1[12f/12f], are viable and fertile in a mixed genetic background. However, they exhibit effects typical of reduced NOTCH1 signaling in T cell development. Thymus size and thymocyte numbers were reduced ~50%, and T cell progenitors, CD4 or CD8 single-positive (SP) and CD4/CD8 double-positive (DP) T cells were reduced in thymus. In addition, Notch1[12f/12f] thymocytes exhibit reduced expression of activated NOTCH1 (NICD), and reduced levels of Notch targets Hes1 and Dtx1. Binding of Notch ligand DLL1 to thymocytes is also reduced. The defective T cell phenotype is cell autonomous following transplantation of Notch1[12f/12f] bone marrow. Therefore, loss of one critical fucose in EGFI2 of NOTCH1 significantly weakens signaling via NOTCH1, even though O-fucose is probably present on all combinations of Notch receptors and ligands, gives more severe reductions in lymphoid and myeloid cells than deletion of Pofut1 in HSC (Yu et al., 2015), suggesting that other O-glycans on Notch receptors might support Notch signaling in the absence of O-fucose glycans. We recently addressed this question by generating mice lacking Eogt and O-GlcNAc glycans as well as being conditionally deleted for Pofut1 in HSCs, as described below (Tanwar and Stanley, 2022).

The question of whether loss of O-Fuc from EGFI2 in NOTCH1 due to conversion of Thr to Ala or the amino acid mutation itself causes the T cell phenotype was not addressed in the mouse mutant. However, it was previously investigated in cell-based Notch signaling assays. In these experiments, the Thr in NOTCH1 EGFI2 was changed to Ala to abrogate O-Fuc addition or, alternatively, changed to Ser which accepted O-fucose (Shi et al., 2007). The Thr to Ala mutation in EGFI2 inactivated NOTCH1 signaling in the cell-based assay, but the Thr to Ser mutation did not, consistent with O-fucose being required in EGFI2 for NOTCH1 signaling. The inhibition of NOTCH1 signaling in the Thr to Ala mutant construct was essentially complete in these experiments. Thus, it was surprising that Notch1[12f/12f] homozygous mice were viable (Ge and
proliferation and causes them to differentiate prematurely to CD8 double-negative 3 (DN3) T cell progenitors inhibits their development (Tsukumo et al., 2006). Loss of Notch1[12f/12f] homozygotes died at E11.5 (Varshney et al., 2019). It seems that a modifying activity in the initial mixed genetic background rescued Notch1[12f/12f] from embryonic lethality.

These findings highlight the complexities of interpreting the results of point mutations that preclude a glycosylation reaction. In another example, a mutation converting an Asn which receives an N-glycan in the enzyme POFUT1 to Leu was found to reduce POFUT1 activity. However, substitution of the Asn with Gln that also could not receive an N-glycan, restored POFUT1 activity (Takeuchi et al., 2018b). Therefore, certain amino acids at that position are required for POFUT1 activity, but the N-glycan attached to Asn is not required for POFUT1 to be active. In another example, mutation of Thr to Ala in the EGF repeat of Cripto inhibits Cripto signaling. However, mutation to Ser, which could receive an O-fucose, also inhibits Cripto signaling, leading to the conclusion that the Thr, and not the O-fucose, is necessary for Cripto signaling (Shi et al., 2007). The take-home message from these experiments is that all caveats should be explored to enable interpretation of mutant phenotypes in relation to glycosylation status.

**Fringe GlcNAc-transferases in lymphopoiesis and myelopoiesis**

The first investigation of roles for Fringe GlcNAc-transferases in T cell development showed that overexpression of Lfng under the control of the lck promoter that is initially expressed in T cell progenitors, blocks T cell development and leads to a large population of B cells in the thymus (Koch et al., 2001). This result is consistent with numerous subsequent experiments showing that B cells and myeloid cells are generated from thymic T cell precursors blocked in Notch signaling by a variety of strategies (Brandstader and Maillard, 2019). Most interesting is the basis of the block caused by overexpressing Lfng in thymocytes, which might be expected to enhance rather than block Notch signaling because LFNG modification of Notch receptors generally enhances signaling induced by DLL ligands (Hicks et al., 2000). Elegant experiments showed that mature thymic T cells overexpressing Lfng compete with early T cell progenitors for binding to DLL4-expressing thymic stroma, and thereby prevent access of T cell progenitors to Notch ligands that stimulate Notch signaling and promote differentiation along the T cell lineage (Visan et al., 2006). Overexpression or loss of Lfng in fetal liver-derived HSC confirmed that Lfng is required for optimal T cell development (Tsukumo et al., 2006). Loss of Lfng from CD4/CD8 double-negative 3 (DN3) T cell progenitors inhibits their proliferation and causes them to differentiate prematurely to CD4/CD8 double-positive (DP) T cells (Yuan et al., 2011). Thus, extension of O-fucose on NOTCH1 in pre-T cells by LFNG enhances Notch ligand binding to Notch receptors and Notch signaling, thereby prolonging proliferation of the pool of DN3 T cell progenitors and promoting the production of mature thymic T cells. The role of LFNG is therefore to enhance the strength of Notch signaling in T cell progenitors, a necessary boost required for optimal production of T cells in thymus (Yuan et al., 2011). However, overexpression of Lfng under the lck promoter could not fully rescue reduced T cell production in the Notch1[12f/12f] thymus (Visan et al., 2010). This result indicates that extension of the O-fucose at EGFL12 of NOTCH1, regardless of potentially enhanced extension of O-fucose at other EGF repeats due to prolonged overexpression of Lfng, is required for optimal Notch signaling in T cell progenitors. In these experiments, Lfng expression was manipulated only in Notch receptor-expressing T cells. Requirements of Lfng for Notch ligand functions in thymic stroma were not addressed. However, there are cellular contexts in which effects of Fringe modification of canonical Notch ligands have been identified in other systems (for example (Bochter et al., 2022) and (Kadur Lakshminarasimha Murthy et al., 2018)), and it will be important to examine this issue and the role of each FNG during hematopoiesis in future.

Roles for each of the Fringe GlcNAc-transferases in T and B cell development were revealed in mice inactivated for all three Fng genes compared to mice expressing a single Fng gene from one allele (Song et al., 2016). Loss of all three Fringe GlcNAc-transferases leaves Notch receptors and Notch ligands with EGF repeats carrying O-fucose that cannot be extended (Figure 2A). Triple knockout (Fng tkO) mice have reduced DLL4-Fc binding to DN T cell progenitors, and reduced frequencies of early thymic progenitors (ETP), DN2 T cell progenitors, and double-positive (DP) T cells (Song et al., 2016). Increased frequencies of CD4 and CD8 single-positive (SP) T cells in the thymus were a novel characteristic of Fng tkO thymus that could indicate delayed exit of mature SP T cells from the thymus. The Fng tkO phenotype is transferred cell-autonomously by bone marrow. Substantial rescue of T cell development is achieved in mice expressing only Lfng, only Mfng, or only Rfng from a single allele. Thus, each Fng contributes to Notch signaling in thymic T cells and promotes optimal T cell development.

In spleen, both LFNG and MFNG are required for MZ B cell development (Tan et al., 2009). Deficiency of either one reduces the number of MZ B cells in spleen. In mice expressing no Fng genes, there is a reduced frequency of MZ B cells as expected, and a corresponding increase in Fo B and MZ P precursor B cells. CD4+ and CD8+ splenic T cells are also decreased. By contrast, granulocyte numbers are increased (Song et al., 2016). All changes are consistent with reduced Notch signaling and show that Fringe modification is necessary for optimal T and B cell and granulocyte production. In terms of individual Fng genes, Lfng was able to rescue the frequency of T and MZ B cells.
in the absence of both Mfg and Rfg. Mfg or Rfg expressed from a single allele accomplished a less complete rescue (Song et al., 2016). There are also functional consequences for T cell responses in mice lacking all Fringe activities. Fng tKO splenocytes stimulated by anti-CD3/CD28 beads or lipopolysaccharide (LPS) or Concanavalin A exhibit reduced proliferation (Song et al., 2016). In addition, naïve T cells and effector memory T cells were reduced in Fng tKO spleen.

**O-GlcNAc glycans in hematopoiesis**

Mice lacking Eogt and O-GlcNAc are viable and fertile but exhibit mildly defective Notch signaling during retinal angiogenesis (Sawaguchi et al., 2017). This laboratory is investigating effects of deleting Eogt and O-GlcNAc glycans on hematopoiesis, lymphopoiesis and myelopoiesis in Eogt null mice. In Eogt[−/−] thymus, T cell progenitors and DP T cell numbers were reduced, while SP T cells were slightly increased (Tanwar and Stanley, 2022), as observed when all three Fng genes are deleted (Song et al., 2016). Consistent with Notch signaling being inhibited due to the loss of Eogt and O-GlcNAc glycans, B cells and myeloid cells were increased in thymus, and Notch target genes Hes1 and I2ra were reduced in expression. In spleen, CD4+ and CD8+ T cells were not reduced, but NK T cells were reduced along with myeloid cells. Surprisingly, B cell subsets were slightly increased. The Eogt null hematopoietic phenotype was largely cell-autonomous following bone marrow transfer.

The fact that loss of Eogt caused effects on T and B cell development consistent with reduced Notch signaling suggested that O-GlcNAc glycans might support Notch signaling in the absence of Pofut1 and O-fucose glycans. This hypothesis was tested by deleting Pofut1 via Vav1-Cre in HSCs, in the presence and absence of Eogt. Deletion of Pofut1 in HSCs caused myeloid hyperplasia and a drastic reduction in T cell development and MZ B cell production, as described previously (Yao et al., 2011; Yu et al., 2015). The additional deletion of Eogt enhanced the Notch-defective phenotype in bone marrow, thymus and spleen (Tanwar and Stanley, 2022). Thus, EOGT and O-GlcNAc glycans are required for optimal Notch signaling in the development of myeloid and lymphoid cells. In addition, O-GlcNAc glycans promote Notch signaling in the absence of O-fucose glycans and partially rescue HSCs and descendants that lack Pofut1.

**Congenital diseases affected in EGF repeat modification by O-glycans**

Congenital disorders of glycosylation (CDG) are rare in human populations, but there are many distinct diseases. At least count, congenital mutations have been reported in >140 different genes involved in glycosylation (Sosicka et al., 2021; Lebeer et al., 2022). Of the glycosyltransferases that modify Notch EGF repeats and promote hematopoiesis, deleterious mutations compatible with life in humans have been identified in POFUT1, LFNG, and EOGT. Mutations in POFUT1 cause an autosomal dominant disease termed Dowling Dego Disease 2 (DDD2) which causes skin hyperpigmentation and follicular skin lesions (Li et al., 2013). However, genome sequencing and mosaic analysis have identified mutations in POFUT1 that are not classified as a classical DDD2 phenotype. A recessive mutation in a person with microencephaly and developmental delay was found to correlate with weak POFUT1 activity (Takeuchi et al., 2018b). Another recessive POFUT1 mutation expands the DDD2 phenotype with new features such as dermatitis (Atzmony et al., 2019). Given the requirement for POFUT1 for lymphopoiesis and myelopoiesis described above, patients with weak POFUT1 may have deficiencies in innate and adaptive immunity. Skin lesions observed in these patients may reflect reduced activity of the immune system in skin (Nguyen and Soulika, 2019).

Humans with inactivating mutations in LFNG have Spondylocostal Dystosis Type 3 (SCDO3), an autosomal recessive disease that gives rise to malformation of the skeleton during embryogenesis (Sparrow et al., 2006; Dunwoodie, 2009). Deletion of Lfng in the mouse causes severe skeletal damage with loss of the tail (Evrard et al., 1998; Zhang and Gridley, 1998), and may be embryonic lethal, depending on genetic background (unpublished observations). LFNG promotes T and MZ B cell development, but is partially compensated for by MFNG and Rfng (Tan et al., 2009; Song et al., 2016). Consequently, effects of the loss of LFNG activity on immune cell development would be expected to be relatively minor in humans, and thus impacts on the immune response of SCDO3 patients would also presumably be minor.

Autosomal recessive EOGT mutations in humans give rise to a disease termed Adams-Oliver Syndrome type 4 (AOS4), which is characterized by aplasia cutis congenita of the scalp, limb and digit malformations, and in some cases, vascular and cardiac defects (Schröder et al., 2019). The O-GlcNAc-transferase activity of EOGT containing AOS4 mutations is greatly reduced (Ogawa et al., 2015). Considering that AOS4 disease varies in severity and that AOS4 patients may live to adulthood, and given the defective T and altered B cell development observed in Eogt null mice (Tanwar and Stanley, 2022), EOGT mutations would be expected to affect immune cell development in humans with AOS4, and potentially to compromise certain immune responses. Further experiments on innate and adaptive immune responses in Eogt null mice will assist in predicting immune response concerns in AOS4 patients.

**Lymphoid and myeloid malignancies with altered Notch signaling**

Notch signaling was first discovered in mammals in acute T lymphoblastoid leukemia (T-ALL) which arises in many cases from a chromosomal translocation that causes constitutive...
activation of NOTCH1 (Ellisen et al., 1991). Subsequently, activating mutations in the NOTCH1 gene were found to cause >50% of T-ALLs (Weng et al., 2004). A proportion of these mutations occur in the NOTCH1 heterodimerization (HD) domain just above the plasma membrane (Figure 1) and render NOTCH1 active independently of Notch ligand stimulation. However, Notch ligands may theoretically stimulate such activated NOTCH1 mutants, since the ECD with its attached O-glycans is present. Another class of NOTCH1 mutations in T-ALL occurs in the PEST domain and functions by prolonging the life of NICD in the nucleus (Weng et al., 2004). In this case, Notch ligand induces Notch signaling which is sustained due to the lack, or inactivation of, the PEST domain on NICD. Several other types of leukemia also arise from PEST domain mutations in NOTCH1, including chronic lymphocytic leukemia (CLL), and Diffuse Large B cell lymphoma (Siebel and Lendahl, 2017). While there have been no reports of an involvement of O-glycan synthesis in leukemogenesis or other blood disorders with disrupted Notch signaling, amplification of the POFUT1 gene has been observed in certain cases of myeloid malignancy (Mackinnon et al., 2010). Thus, loss of the long arm of one copy of chromosome 20, that occurs in cases of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and is presumed to assist leukemogenesis by removal of a tumor suppressor-like gene, is often accompanied by amplification of the remaining portion of chromosome 20 (20q11.2). The POFUT1 gene resides in the smallest amplified domain at 20q11.21, along with the HCK, TM9SF4 and PLAGL2 genes. Investigation of a functional role for POFUT1 gene amplification in models of AML and MDS might reveal enhanced Notch signaling due to increased O-fucosylation of Notch receptors, or ligands. A similar amplification of chromosome region 20q11.21 is seen in colon cancer (Chabanais et al., 2018). However, the website Tumor Portal sponsored by the BROAD Institute (http://www.tumorportal.org/), which reports expression levels and mutations across many cancer types, notes that neither POFUT1, LFNG nor EOGT are “near significance” for mutation or expression changes in any lymphoid or myeloid tumor type.

Discussion

The development of HSCs, the many HSPCs, myeloid, and lymphoid cells reflects a complicated set of lineage decisions requiring co-ordination at the molecular and cellular levels. Notch signaling is a key regulator of several stages of differentiation through different combinations of Notch receptors, Notch ligands and glycosyltransferases that modify extracellular domain EGF repeats (Figure 1). The focus of this review is on Notch receptors, ligands and glycosyltransferase genes that have been manipulated to uncover specific roles in hematopoiesis, myelopoiesis and lymphopoiesis for regulation by glycosylation. Table 1 summarizes key experimental systems and findings discussed in the text. NOTCH1 is essential for T cell development and DLL4 is required, but Dll4 deletion in thymic stroma can be rescued by Dll1. NOTCH2 is essential for MZ B cell development and DLL1 is also required. It is not known whether DLL4 could substitute for DLL1 in splenic stroma. JAG1 and JAG2 have roles in the generation of HSCs from hemogenic endothelium and the glycosyltransferases have various roles. Deletion of Pofut1 is embryonic lethal (Fernandez-Valdivia et al., 2011), and to date there have been no reports on the consequences of conditionally deleting Pofut1 and O-glucose glycans in HSCs, or other immune cells, or stromal cells. However, the effects of knocking out Pofut1, Lfng, Mfng, Rfng and Eogt on myeloid and lymphoid cell development have been investigated. Deletion of Pofut1 in HSCs causes altered generation of B cells, myeloid cells, and thymus-seeding progenitors in bone marrow. Pofut1 null T cells that enter the thymus are mainly converted to B cells and myeloid cells, and T cell development is blocked. A similarly dramatic phenotype is observed when Lfng is overexpressed in thymic T cells. Loss of Lfng causes a relatively mild reduction in T cell development which is ameliorated by the presence of Mfng and/ or Rfng. In spleen, Lfng and Mfng are required together for optimal MZ B-cell development. Mice lacking Eogt exhibit altered cell numbers in bone marrow B cells and myeloid cells, thymic T cells and MZ B cells in spleen. As structural methods develop it should become possible to analyze the actual changes in O-glycan occupancy and extension among Notch receptors in mouse models. For now, the combined data suggest that sugars added to the EGF repeats of Notch receptors and ligands play a part in optimizing the generation of appropriate numbers of cells during hematopoiesis, myelopoiesis and lymphopoiesis. Reduced ligand binding to progenitor cells identifies one mechanism for reduced Notch signaling strength. Consistent with this, reduced ligand binding is observed in DN T cells expressing NOTCH1[12f] in which there is no O-fucose attached to EGF12, or no expression of LFNG, MFNG or RFNG. LSK cells in bone marrow show reduced Notch ligand binding when POFUT1 is absent. However, loss of EOGT does not significantly affect soluble Notch ligand binding to DN T cells, although Notch signaling is reduced, indicating that binding strength may be impaired. Since glycosyltransferases that modify Notch promote immune cell development, people with deleterious mutations in one of these glycosyltransferase genes may have immune response defects. Functional consequences were observed in responses of activated T cells lacking Fringe activity, but many more studies are needed to uncover potential problems with immune responses. In immune cell cancers, inhibition of Notch signaling might be achieved by inhibition of glycosylation. One example of this approach used incorporation of a chemically modified fucose into EGF repeats to block Notch signaling (Schneider et al., 2018). A better understanding of how Notch signaling is regulated and
| Gene          | Mouse Models | Bone marrow | Thymus                                                                 | Spleen                                                                 | References                                      |
|--------------|--------------|-------------|------------------------------------------------------------------------|------------------------------------------------------------------------|------------------------------------------------|
| Notch1       | Notch1lox/loxMx1-Cre | BM transfer gave T cell defects, spleen and lymph nodes populated with Notch1<sup>−</sup> cells | T cells greatly reduced. Increased B cells and myeloid cells | No effects on B cells, erythroid or myeloid cells | Radtke et al. (1999), Wilson et al. (2001) |
| Notch2       | Mx1-Cre | BM transfer of T cell phenotype | -50% less thymocytes, reduced T cells. No increase in B-cells. | Not investigated | Ge and Stanley, (2008) |
| Notch1, Notch2 | HSC deletion by CRISPR/Cas9 | No effects on T cell development | Largely abolished MZ B cells and precursors | Not applicable | Romero-Wolf et al. (2020) |
| RBPJ         | RBPJfl/flMx1-Cre | Dysregulated HSPC differentiation | Reduced T cell development Increased B cells and myeloid cells | No change in B cells | Han et al. (2002), Yu et al. (2015) |
| Dll1         | Dll1lox/loxMx1-Cre | No change in hematopoiesis | No change in T cell development | MZ B cells largely absent | Hozumi et al. (2004) |
| Dll4         | Dll4<sup>Mfox</sup>Foxn1-Cre | Reduced LMPP/MPP4 progenitors Increased GMP | Reduced T cell development Increased B cells and myeloid cells | Disregulated B cell development Myeloid hyperplasia | Chen et al. (2019), Lakhani and Rathinam, (2021) |
| Jag1         | Jag1lox/loxMx1-Cre | No effect on HSC or HSPC | No phenotype | No phenotype | Mancini et al. (2005) |
| Jag1<sup>Mox</sup> | VE-cadherin-Cre | Reduced LT-HSC, LSK cells increased stress sensitivity (LSK) | Not investigated | Not investigated | Poulos et al. (2013) |
| Jag2         | Jag2<sup>Mox</sup>VE-cadherin-Cre | Block in HSC generation from AGM | Not investigated | Not investigated | Gama-Norton et al. (2015) |
| Lfng         | PrLfng transgenic (Proximal Lck promoter) | Not investigated | T cell development blocked at pro-T stage. Increased pro-B and more mature B cells | No apparent effects | Koch et al. (2001), Visan et al. (2006) |
| Lfng, Jag1   | PrLfngNotch1[12f][12f] | Not investigated | Ameliorated PrLfng phenotype | Not investigated | Visan et al. (2010) |
| Lfng, Jag2   | PrLfng<sup>Mfox</sup>,<sup>Mfox<sup>+</sup></sup>Lfng<sup>+</sup>,<sup>Mfox</sup> dKO | BM transfer of spleen phenotype from all mutants | Not investigated | Reduced MZ B cells in single and double KO. Lfng and Mfng complementary | Tan et al. (2009) |
| Lfng,Mfng, Rfng | Fng<sup>+</sup>KO | BM transfer of thymus and spleen phenotypes | Reduced T cell development No increase in B cells Corrected by each Fng alone | Reduced MZ B and Fo B cells. Increased MZP, neutrophils and NK T cells. Reduced activation of T cells | Song et al. (2016) |
| Pofut1       | Pofut1<sup>Mfox</sup>Mx1-Cre | Myeloid hyperplasia, reduced B cells. Increased GMP, reduced CMP, MEP. BM transfer of thymus and spleen phenotypes | Blocked T cell development Increased B cells and myeloid cells | Reduced T cells, MZ B cells Increased myeloid cells | Yao et al. (2011), Yu et al. (2015), Tanwar and Stanley, (2022) |
| Eogt         | Eogt<sup>+</sup> | Increased B cells and myeloid cells. Phenotype partially transferred by BM | Reduced T cell development Increased B cells and myeloid cells | Increased B cells Reduced NK T cells and myeloid cells | Tanwar and Stanley, (2022) |
| Eogt, Pofut1 | Eogt<sup>+</sup>Pofut1<sup>Mfox</sup>Vav1-Cre | Increased ST-HSC, LSK, HSPC, GMP Reduced MPP, CLP, CMP, MEP, T cells, B cells | T cell development abolished Increased B cells, myeloid cells, NK cells | Reduced Fo B, MZ B, mature B cells and T cells. Increased MZ P and myeloid cells | Tanwar and Stanley, (2022) |
becomes dysregulated will enable promotion or inhibition of signaling in order to optimize immune responses in humans. Other signaling pathways such as those directed by cytokines or Notch pathway contains one or more EGF repeats with O-glycans required for the function of the parent molecule.

Author contributions

AT wrote a proposed draft and PS wrote the manuscript. AT and PS edited the manuscript.

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Conflict of interest

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Lunatic fringe controls T cell differentiation through modulating notch signaling. 

**Molecular mechanisms of notch signaling in lymphoid cell lineages**

Differentiation of lymphoid cells involves the activation and suppression of notch signaling, which is crucial for the development of lymphocytes. The role of glycosyltransferases in modulating notch signaling in HEK293T cells reveals insights into development and disease. 

**NF-κB and beyond.**

NF-κB is a transcription factor that plays a key role in the immune response and inflammatory processes. The regulation of NF-κB expression and activity is essential for the proper development and function of the immune system. 

**Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for delta-like Notch ligands**

Developmental delay with cardiac and vascular features is observed in humans with mutations in the LUNATIC FRINGE gene. 

**Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways.**

Protein O-fucosyltransferase 1 regulates the balance of notch signaling and hematopoiesis. 

**Activation of NOTCH1 in human T cell acute lymphoblastic leukemia.**

Activated notch signaling is implicated in the development and transformation of human hematopoietic cells. 

**Notch symptonic regulation of notch signaling by different O-glycans promotes hematopoiesis.**

Different O-glycans play a critical role in the regulation of notch signaling, which is essential for the development and function of hematopoietic cells. 

**Protein O-fucosyltransferase 1 (Pofut1) regulates lymphoid and myeloid homeostasis through modulation of Notch receptor ligand interactions.**

Pofut1 is involved in the regulation of Notch signaling and hematopoiesis. 

**Lunatic, manic fringe cooperatively enhance marginal zone B cell precursor competition for delta-like 1 in splenic endothelial niches.**

Lunatic and manic fringe proteins work together to enhance the competition for delta-like 1 ligands in splenic endothelial niches. 

**Synergistic regulation of notch signaling by different O-glycans promotes hematopoiesis.**

Synergistic regulation of notch signaling by different O-glycans is associated with hematopoiesis. 

**Disruption of POGLUT1 and POFUT1 reduces viability of Notch1[12f/12f] mice.**

Disruption of Pofut1 reduces viability in Notch1[12f/12f] mice, highlighting the role of Pofut1 in notch signaling. 

**Regulation of pre-TCR signaling during thymocyte beta-selection in vivo.**

Regulation of pre-TCR signaling is important for thymocyte development and beta-selection. 

**Mechanisms of notch signaling in megakaryocyte-erythroid progenitor cells (MEPs) and beyond.**

Mechanisms of notch signaling in MEPs and beyond are crucial for the development of hematopoietic cells.