ROLES OF *O*-FUCOSE MOLECULES IN NOTCH SIGNALING AND HEMATOPOIESIS

by

DAVID C. YAO

Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

Thesis Adviser: John B. Lowe, M.D. and Robert Fairchild, Ph.D.

Department of Pathology

CASE WESTERN RESERVE UNIVERSITY

August, 2011

CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

David C. Yao

candidate for the Doctor of Philosophy degree *.

(signed) Clark Distelhorst, M.D.
(chair of the committee)

Robert Fairchild, Ph.D.

Lan Zhou, M.D., Ph.D.

Clive Hamlin, Ph.D.

(date) August 11th, 2010

*We also certify that written approval has been obtained for any proprietary material contained therein.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I want to first thank my advisor, Dr. John Lowe, for his support in my pursuit of a doctorate degree. He challenged me to formulate hypotheses and gave me the opportunity to solve these problems independently. He showed me ways to present my findings in a clear and organized fashion. His teaching and guidance have helped me develop the necessary traits to have a successful career as a physician scientist.

I want to give a special thanks to my co-advisor, Dr. Robert Fairchild, who exhibits mastery with current methods and concepts in immunology. His energy and enthusiasm for science and teaching make him a true role model. His thoughtful suggestions and constructive criticisms have made the completion of my dissertation possible. I am grateful to him for his help in this regard.

I also want to express my appreciation wholeheartedly to Dr. Lan Zhou, for providing a wonderful research and learning opportunity and whose continual excitement in searching for scientific answers throughout our study made a lasting impression. Her passion for science is contagious. Her availability for spirited discussions regarding all aspects of science made mundane laboratory tasks exciting. Because of her active engagement, my research experience was exceptionally satisfying.

I would like to thank my other committee members, Dr. Clark Distelhorst and Dr. Clive Hamlin, and several fellow researchers, Jay Myers, Bronislawa Petryniak, Dr. Jeongsup Shim and Yungshuai Huang, for all the time commitment, constructive criticisms, insightful recommendations, ancillary support, technical knowledge and demanding questions.

Last but not least, I want to extend my gratitude to three extraordinary people in my life: my wife Jennifer Eads, my clinical mentor, Dr. Jerry Goldstone and my mother Feng-feng Shieh for their uplifting encouragement while embarking on my dissertation project. Armed with their continual support and confidence in me, I was able to pursue my research interests with ease. Without their love and support this dissertation would not have been completed.

LIST OF APPREVIATIONS

5-FU	5-FluoroUracil
APC	Antigen Presenting Cell
BM	Bone marrow
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CCR	Chemokine (C-C motif) Receptor
CD	Cluster of Differentiation
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CSL	CBF-1/RBP-Jκ in mammals, Suppressor of Hairless in Drosophila, and Lag in C. elegans
Dll	Delta Like
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DN	Double Negative
ECN	Extracellular Domain of Notch
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescence Protein
ELISA	Enzyme-linked Immunoabsorbent Assay
ESC	Embryonic Stem Cell
EYFP	Enhanced Yellow Fluorescence Protein
ETP	Early Thymic Progenitor
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FSC	Forward Scatter
FX	3,5 epimerase/4-reductase
Flt3	FMS-like Tyrosine Kinase 3
GalNAc	N-acetylgalactosamine
GDP	Guanosine diphosphate
GMD	GDP-mannose 4,6-dehydratase
GMP	Granulo-monocytic Progenitor
GluNAc	N-acetylglucosamine
GSI	Gamma Secretase Inhibitor
HEK	Human Embryonic Kidney
hIgG	Human Immunoglobulin G
HPC	Hematopoietic Progenitor Cell
HRP	Horseradish Peroxidase
HSC	Hematopoietic Stem Cells
ICN	Intracellular Domain of Notch
IL	Interleukin
J1	Jagged1
J2	Jagged2
Lfng	Lunatic Fringe

LSK	Hematopoietic Stem Cells Carrying Surface Markers: Lin- Sca-1+ c-Kit+
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
Mfng	Manic Fringe
MPP	Multi-potent Progenitor
Mx-Cre	Myxovirus Resistance 1-driven Causes Recombination Recombinase
MZB	Marginal Zone B cells
N1	Notch1
N-CAM	Neural Cell Adhesion Molecule
PCR	Polymerase Chain Reaction
Pofut1	Protein O-fucosyltransferase 1
Poly (I:C)	Polyinosinic:polycytidylic Acid
PSA	Polysialic acid
PSGL-1	P-selectin glycoprotein glycan-1
Rfng	Radical Fringe
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCF	Stem Cell Factor
SD	Standard Deviation
SLeX	Sialyl-LewisX
SP	Single Positive
SSC	Side Scatter
TCR	T-cell Receptor
TNF	Tumor Necrosis Factor
TSR	Thrombospondin-like Repeat
WT	Wild-type

Roles of *O*-fucose Molecules in Notch Signaling and Hematopoiesis

Abstract

by

DAVID C. YAO

In mammals, fucose molecules are frequently present as a terminal modification of glycans. To elucidate the biological roles of fucose molecules, we previously characterized mice engineered to be conditionally deficient in all fucosylation. These mice yield phenotypes, including growth retardation, infertility, thymic hypoplasia and myeloproliferation. Functional analyses reveal that fucose-dependent thymic hypoplasia and myeloproliferation phenotypes are a result of defective Notch activity. Notch signaling is a conserved pathway that regulates cell fate determination. The extracellular domain of Notch is decorated with multiple *O*-fucose molecules, of which the additions are catalyzed by Protein *O*-fucosyltransferase-1 (Pofut1). These fucose-containing glycans have been demonstrated to be critical in modulating transactivation of Notch. To elucidate the role of *O*-fucose-dependent Notch activity in post-natal hematopoiesis, we studied mice with conditional inactivation of Pofut1. Adult animals bearing the homozygous Pofut1-deficient allele exhibits myeloproliferation, faulty marginal zone B-cell development and thymic hypoplasia in both cell-autonomous and non-autonomous

fashions. Similarly, Pofut1-deficient bone marrow progenitors display robust myeloid development but fail to differentiate into T lymphocytes while co-culturing with Notch-ligand-expressing stromal cells *in vitro*. Both myeloproliferation and deficient mature T cell traits are reversed when Pofut1-deficient progenitors are reconstituted with constitutively active Notch1, which illustrates the critical role of Notch *O*-fucosylation in regulating hematopoietic homeostasis. Although *O*-glycans are essential for Notch function, it is unclear whether all *O*-fucose molecules are important. To address this issue, we used cell-based assays to assess the binding efficiency of the Notch ligands Delta-like-1, Delta-like-4, Jagged1 and Jagged2 to mouse Notch1 and the expression levels of surface Notch1 as a function of site-directed deletion of *O*-fucosylation sites thought to contribute to Notch biology (EGF domains 8, 9, 12, 24, 26 and 27). Binding and surface expression data from these studies are then correlated with Notch ligand-mediated signal transduction. These studies allow us to conclude that while multiple *O*-fucosylation sites modulate the surface expression of Notch1, *O*-fucose glycans in EGF9 and EGF12 play a primary role in regulating Delta-like-1, Delta-like-4 and Jagged2-mediated Notch binding and signaling. Together, our studies highlight the effects of multiple roles of *O*-fucose moieties on Notch activity and blood cell formation homeostasis.

CHAPTER I

Introduction

Complex Carbohydrates, Glycoconjugates and Fucose Biology

Carbohydrates, along with nucleic acids, proteins and lipids, are critical components of all living organisms. These molecules play a wide spectrum of essential roles, including energy storage, structural framework of DNA and RNA, elements of bacterial and plant cell walls and many other biologic functions while attached to proteins and lipids. The most fundamental unit of a carbohydrate is the monosaccharide, which is composed of either a ketone or aldehyde with two or more hydroxyl groups. Complex carbohydrates consist of two or more monosaccharides joined by one or more glycosidic bonds, which exist in either linear or branched fashion. Depending on the number of monosaccharide units present, complex carbohydrates can be further categorized into oligosaccharides and polysaccharides.

While polysaccharides exist as standalone branched or unbranched polymers, oligosaccharides are often bound to secreted or cell surface-expressing proteins to form glycoconjugates. These complex carbohydrates are attached to the nitrogen of asparagine residues or the oxygen of serine or threonine residues by *N*-linked or *O*-covalent bonds, respectively. *N*-linked oligosaccharides are composed of a universal pentasaccharide core including three mannose and two *N*-actylglucosamine molecules. Additional sugars are then appended to this core structure to form three different patterns: *oligomannose*, where only mannose molecules are attached to the core, *complex*, where the core structure is modified by different sugars starting with *N*-acetylglucosamine (GluNAc) and *hybrid*, where both oligomannose and complex sugars are attached to the core structure (1). *O*-

glycans are commonly linked to the hydroxyl group of serine or threonine residues via *N*-acetylgalactosamine (GalNAc). This basic unit can then be extended into a wide spectrum of core structures (2-5). In addition to *O*-GalNAc, several other types of *O*-glycans have been identified, including fucose, galactose, glucose, mannose and GluNAc (6).

The biosynthesis of protein-attached oligosaccharides is catalyzed by glycosyltransferases that assemble monosaccharides into glycan chains. All glycosyltransferases share a common ability to transfer an individual monosaccharide moiety from its carrier to the acceptor substrate. These enzymes usually function sequentially where the final product of one enzyme serves as the acceptor substrate of the subsequent reaction. Although glycosyltransferases are primarily responsible for the elongation of glycans, some function by removing monosaccharides from the existing chain to form intermediates, which can then be processed further (7). Glycoconjugates can also undergo a number of structural modifications, including phosphorylation, sulfation, acetylation, methylation and many others (8), each of which is catalyzed by unique enzymes (9-11). Considered together with the differential expressions of glycosyltransferases and glycan-modifying enzymes in a cell-specific and temporally regulated fashion, the final oligosaccharide ensemble is often diverse.

Given the structural complexity inherent in glycoconjugates, these complex carbohydrates likely carry a rich body of biological information. To date, biological roles have been assigned to glycans range from dispensable to those that are essential to development and survival. In terms of structural roles, glycosylation is involved in the synthesis, folding and trafficking of glycoproteins (12-14). For example, deficient

phosphorylation of mannose residues of N-glycans on various lysozymes leads to mislocalization of these enzymes, which subsequently results in the accumulation of lysosomal substrates and impaired cellular functions (15). Glycans can also positively or negatively regulate the interaction of proteins and thus modulate the subsequent molecular events. In response to stimuli, platelet-selectin (P-selectin) is trafficked to the surface of endothelial cells where it binds to the sialyl LewisX terminal glycan motif on P-selectin glycoprotein ligand-1 (PSGL-1), a trans-membrane glycoprotein expressed on all leukocytes. The recognition of P-selectin to PSGL-1 recruits neutrophils and monocytes to the sites of inflammation (16). While the sialyl-LewisX (SLeX) glycan domain enhances the interaction of P-selectin and PSGL-1, the polysialic acid (PSA) moiety attached to neural cell adhesion molecule (NCAM) inhibits the homophilic binding (17). This interference of homophilic binding between NCAM mediated by PSA leads to reduced cell adhesion and enhanced cell migration (18, 19). In addition to functional roles within an organism, glycoconjugates facilitate the interactions between species. For instance, *Plasmodium* species invade host erythrocytes via the binding of self surface proteins, such as erythrocyte-binding antigen-175, to clusters of sialylated *O*-glycans attached to glycophorin A, a surface expressing glycoprotein on erythrocytes (20, 21). Together, with evidence from recent reports, biological roles for glycans cross a wide spectrum.

Given the diverse biological roles of glycans, it is not surprising that these molecules have been associated with a wide variety of diseases in humans, ranging from congenital disorders to acquired diseases. Many genetic defects of glycosylation in humans have been discovered recently, most of which have heterogeneous clinical

manifestations with multiple potential organs involved. Defects in the components of the glycan synthesis machinery have been identified as the pathogenesis of these rare inherited disorders (22). Acquired changes in glycans have also been implicated in a wide spectrum of non-hereditary diseases. These diseases are often prevalent. For instance, the expression of SLeX has been credited to the formation of reperfusion injury (23, 24), atherosclerosis (23, 25, 26), acute respiratory distress syndrome (27), inflammatory skin disease (23), inflammatory bowel disease (23, 28) and hematogenous carcinoma metastasis (29-31). Altered glycosylation other than SLeX in acquired diseases is also well documented. For example, autoimmune IgM antibodies against glycan epitopes on erythrocytes give rise to cold agglutinin disease, which leads to hemolytic anemia (32). In yet another example, the truncation of *O*-glycans on serum IgA1 results in IgA nephropathy (33) while markedly increased branching of *O*-glycans of CD43 on T cells may contribute to symptoms observed in Wiskott - Aldrich syndrome, a disease characterized by skin eczema, low platelet counts and altered cellular immune responses (34). Although substantial advances have been made in glycobiology, the functionality of many glycoconjugates remains unknown. This stems from the difficulty predicting what glycan structures are involved in specific biological roles. A body of literature suggests that terminal sugar molecules, unusual structures and modifications of the glycans are most biologically relevant (35-37). Many terminal sugars have been found to mediate essential biological roles, of which fucose is included.

L-fucose, unlike other six-carbon sugars, carries a proton at the carbon-6 position and is in the L-configuration. Fucose often exists as the terminal modification of complex glycans. Less frequently, it is found to be attached directly to protein residues. These

fucose-containing glycoconjugates are essential in a variety of normal biological functions, including but not limited to, ABO blood group antigen determination, host and microbe interaction, leukocyte adhesion and proper activation of molecular events in different contexts (Table 1-1). Furthermore, many human diseases, including cancer (29, 30, 38), rheumatoid arthritis (39, 40) and recurrent infections (41, 42) have been associated with fucosylated glycans. Because of the diversity of fucosylated glycans, it is likely that many functions for fucose-containing glycoconjugates have yet to be identified.

Enzymes that are responsible for fucose modifications are fucosyltransferases. Depending on their substrate and linkage specificity, these enzymes can be categorized into different families, each of which may have one or more members. All fucosyltransferases catalyze reactions by transferring fucose from its nucleotide transporter (GDP) to targeted substrates for the formation of final products. To elucidate the roles of fucose in biological function, we have previously studied mice genetically engineered to be conditionally deficient in all fucose modifications. These mice carry a targeted deficiency in the FX locus, which encodes GDP-4-keto-*O*-6-deoxymannose 3,5-epimerase-4-reductase, an enzyme thought to be essential for the constitutive pathway of GDP-fucose synthesis. GDP-fucose is a donor substrate of all the known fucosyltransferases that are essential for the expression of fucose-containing glycoconjugates. *de novo* synthesis of GDP-fucose is illustrated in Figure 1-1. In adult animals, homozygosity for the FX-null allele exhibits a broad spectrum of phenotypes that include infertility, growth retardation, leukocyte adhesion deficiency, inflammatory bowel disease and peripheral neutrophilia (43). More interestingly, it produces a thymic

hypoplasia phenotype unless these animals are provided with a fucose-supplemented diet, which restores fucosylation via a salvage pathway (Figure 1-1). The salvage pathway synthesizes GDP-fucose from fucose originated from the extracellular environment or lysosomes. Further analyses of thymi in the adult FX-null animals, while being placed on a normal diet, reveal that the differentiation process of thymocytes terminates at an early developmental stage. This observation coincides with the published findings that induced loss of function of the Notch1 receptor results in a blockage of thymocyte development at the similar stage (44, 45). Complementary to these observations, hematopoietic stem cells (HSCs) isolated from adult FX-null animals fail to differentiate into mature T cells with the support of Notch ligand *in vitro* (Man, et al., unpublished data). Furthermore, intra-thymic injection of FX-null HSCs reconstituted with constitutively active Notch1 molecules give rise to mature T cells. These findings indicate that the faulty T cell development phenotype in FX-null animals while on normal mouse chow is indeed a result of defective Notch activity.

The canonical Notch signaling pathway

Notch is a series of highly conserved molecular events that is essential for the development of metazoans. Evidence from both molecular and cellular studies has demonstrated that Notch activity plays a crucial role in orchestrating cell differentiation, cell proliferation, apoptosis, and thus normal development of multiple biological systems. Since Notch plays a wide-ranging role in development, it is not surprising that deranged Notch activity has been implicated in a variety of human diseases, including T-cell acute lymphoblastic leukemia (46, 47), Alagille syndrome (48, 49), CADASIL (50), aortic

valve disease (51) and tetralogy of Fallot (52), all of which are characterized by faulty cellular differentiation.

The gene encoding Notch was first cloned in *Drosophila* (53) where the partial loss of function of Notch produces a notch phenotype at the wing margin. Since then, the biology of Notch molecules and the corresponding ligands have been studied in depth. In mammals, Notch receptors are expressed on the cell surface as a hetero-dimeric single-pass transmembrane protein with four homologues, including Notch1, Notch2, Notch3, and Notch4. These Notch molecules recognize one or more surface expressing ligands, including Delta-like-1, Delta-like-3, Delta-like-4, Jagged-1, and Jagged-2. In the canonical Notch signaling pathway, Notch activity is initiated through a cell-cell interaction, where Notch molecules on the surface of one cell bind to Notch ligands on neighboring cells. If this interaction is productive, Notch receptors undergo a conformational change, which instigates a cascade of proteolytic events that a family of membrane-bound metalloproteases (TNF-α converting enzymes) and γ-secretase cleave and release the intracellular domain of Notch (ICN) from its extracellular counterpart. Notch-mediated signal transduction continues when ICN is translocated to the nucleus where it binds to a transcription factor CSL (CBF-1/RBP-Jκ in mammals, Suppressor of Hairless in *Drosophila*, and Lag in *C. elegans*). A conglomerate of ICN and CSL displaces various co-repressors and recruits Mastermind-like family and other co-activators, which subsequently drives the expression of Notch-specific transcripts, including the family of Hes genes, Deltex, Gata-3, and pre-Tα (Figure 1-2).

_O_-fucosylation of Notch receptors

Notch signaling is not simply a binary process where the signal is determined in an “all-or-nothing” fashion upon the pathway in which it is activated. Instead, studies in invertebrates have elucidated a generally accepted notion that the outcome of Notch activity is dependent on the signal strength. This dose dependency mechanism can be attributed to molecular regulations of Notch signaling at multiple levels, including the post-translational modification of Notch receptors by *O*-fucose.

The extracellular domain of Notch is composed of several tandem epidermal growth factor-like (EGF) repeat motifs. Each contains six evolutionarily conserved cysteine residues and serine/threonine residues whose hydroxyl group may be modified by *O*-fucose via a covalent bond. These *O*-fucose molecules reside between the second and the third cysteine residues of an EGF-repeat module that contains a consensus peptide sequence $C_2X_{4\text{-}5}(S/T)C_3$, where X represents any amino acid residues (54, 55). Addition of *O*-fucose is catalyzed by Protein *O*-fucosyltransferase 1 (Pofut1) in the endoplasmic reticulum (56). In addition, Pofut1 homolog in *Drosophila* has a chaperone activity, which regulates the normal surface expression of Notch molecules (57). Functional deficiency of germ line Pofut1 produces an embryonic lethal phenotype which is similar to that of embryos lacking downstream effectors of the Notch pathway (58). These *O*-fucose moieties can further be elongated by the addition of *N*-Acetylglucosamine, a reaction catalyzed by one of the three fucose-specific β1,3-N-acetylglucosaminyltransferases (Lunatic, Manic and Radical Fringes) in the Golgi apparatus (59). This fucose-dependent post-translational modification of Notch by Fringe proteins is critical in modulating transactivation of Notch through altering the sensitivity of interactions between Notch receptors and their ligands (60). Figure 1-3 illustrates the

enzymatic modifications of the Notch molecules as they are trafficked to the cell membrane.

Hematopoietic Development

As mentioned previously, Notch signaling is critical to the normal development of multiple organs and systems, including hematopoiesis. Throughout life, mature blood cells in adult mammals are replenished by HSCs in the bone marrow. Although it is not without controversy, the notion that differentiation of HSCs follows a hierarchical organization is generally accepted. While positioned at the top of this model, HSCs produce progenitors with a restricted differentiation potential. The entry to lineage-specific development in hematopoiesis is facilitated by the production of multipotent progenitors (MPPs) (61). MPPs can subsequently give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) whose ability to generate mature cells is further confined to lymphoid and myeloid lineages, respectively. CMPs further differentiate into even more lineage restricted progenitors, which give rise to mature myeloid lineage cells, including neutrophils (Figure 1-4).

The development of neutrophils is tightly regulated by growth factors and the bone marrow microenvironment (62). Cytokines that are critical to the proliferation and differentiation of neutrophils include interleukin-3, interleukin-6, granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor (52). In addition to the growth factors, biosynthesis of neutrophil-specific proteins is under the combined and sequential control of transcription factors, including Myb, Gata-1, PU.1 and CCAAT/enhancer-binding protein (C/EBP) (63). These concerted molecular events

regulate the expressions of cell surface receptors and digestive proteins. As neutrophils mature, they acquire a large collection of antimicrobial, digestive and chemotactic proteins, most of which are stored in the cytoplasmic granules and vesicles. These mature granulocytes also express lineage-specific surface markers, such as Gr-1.

The current working model of T cell development is generally accepted (Figure 1-5). The T cell lineage commitment is initiated when CLPs are first released from the bone marrow. While they are circulating in peripheral blood, these progenitors acquire the necessary machinery, such as the surface expression of CD44, CCR9 and PSGL-1 molecules, to migrate into the thymus (64-66). Upon entering the thymus at the cortico-medullary junction, progenitors migrate towards the outer cortical zone where they begin to undergo a maturation process. These cells first develop into the Double-Negative-1 (DN1) stage whose cell surface marker makeup is defined as $CD44^{+}$ $CD25^{-}$ $CD4^{-/lo}$ $CD8^{-}$. While the DN1 cells continuously trek through the outer cortex, they differentiate into DN2 cells ($CD44^{+}$ $CD25^{+}$ $CD4^{-}$ $CD8^{-}$). During the next stage of the cellular transformation, the surface expression of CD44 is repressed in these progenitors, which yields a phenotype $CD44^{lo}$ $CD25^{+}$ $CD4^{-}$ $CD8^{-}$ for DN3 cells. Prior to migrating into the inner cortex, these DN3 cells undergo gene rearrangements at the TCRβ, γ, and δ loci, a process instigated by the expression of both Rag1 and Rag2 genes. Through an unknown mechanism, some cells differentiate into γδ T cells while a large majority of these cells proliferate into the αβ lineage T cells. At the same stage, these TCR^{+} thymocytes further differentiate into DP cells ($TCR^{\alpha\beta+/\gamma\delta+}$ $CD4^{+}$ $CD8^{+}$). These DP cells undergo further differentiation with MHC-antigen-dependent selections and the thymocytes that survive negative selection will mature into either $CD4^{+}$ or $CD8^{+}$ lineage T cells.

During the T cell maturation process described above, the intra-thymic progenitor cells travel through a network of the thymic stromal cells. These stromal cells play an important role in supporting the development of mature T cells. In addition to secreting T lineage-supporting cytokines such as interleukin-7, these non-lymphoid cells express Notch-specific ligands including Delta-like 1, Delta-like 4 and Jagged-2, which trigger Notch signaling through Notch1 molecules on the surface of the progenitors (67-69, 119). The significance of Notch function in T cell development has been documented extensively. The Notch1 deficient bone marrow progenitors terminate their normal T lineage development at DN1 and differentiate into the B cell lineage in the thymus (44). Consistent with this finding, the block of Notch signaling using a high dose γ-secretase inhibitor leads to the termination of T cell development at the DN compartment in fetal thymic organ cultures (70). Using a gain-of-function approach, Pui and colleagues have demonstrated that the enforced expression of intracellular domain of Notch1 drives hematopoietic precursors into T cells in the bone marrow compartment (71). The collection of these data suggests that the activation of Notch1 molecules is necessary for T cell lineage commitment.

Despite intense research, key molecular events that regulate lymphoid and myeloid lineage commitment are still elusive. It has been hypothesized that Notch plays a role in this binary fate decision. Signaling through Notch receptors is critical in the formation of mature T cells (72) although its role in myelopoiesis is slowly emerging. In the presence of constitutively activated Notch1 molecules, myeloid progenitor cell line and freshly isolated bone marrow cells do not differentiate further into mature granulocytes *in vitro* (73-75). Myelopoiesis of hematopoietic progenitors is compromised

while co-culturing with bone marrow stromal cells bearing Notch ligands (76). Bone marrow transplant experiments using both gain-of-function and loss-of-function approaches suggest that Notch signaling favors the lymphoid lineage at the expense of myeloid differentiation (77, 78). Interestingly, sustained activation of Notch1 or Notch2 appears to enhance myelopoietic potential of myeloid progenitors (79, 80). Moreover, deletion of individual component or a combination of components from the Notch ligand-receptor axis did not produce abnormal myeloid development traits (44, 81). Together, functional correlates of Notch activity in the modulation of diversification between lymphoid and myeloid lineages in hematopoietic progenitors requires further examination.

In my dissertation project, the overall goal is to address some of the issues raised thus far by examining the biological role of *O*-fucose glycans in Notch signaling and hematopoiesis. In the following two chapters, I will first present our data demonstrating that *O*-fucose dependent Notch activity is critical for the synthesis of mature blood cells using the murine embryonic stem cell line carrying targeted deficiency in the Pofut1 locus. I will then verify our *in vitro* observations by further characterizing the phenotypic traits in Pofut1-deficient mice. Moreover, I will go over our findings illustrating the novel roles of Notch receptors and ligands in postnatal T lymphoid and myeloid commitment in an *O*-fucose dependent manner. Given that Notch is decorated by numerous *O*-fucose moieties, I will present evidence showing that a subset of these *O*-glycans, through multiple regulatory mechanisms, is required for Notch1 to achieve its normal function in Chapter IV. In Chapter V, I will present our effort to determine cell-type specific Notch1 expression patterns with the corresponding Notch activity in T cell subsets. Through this

series of studies, my hope is to underscore the critical roles for *O*-fucose molecules in Notch biology and thus the lineage commitment in the context of hematopoiesis.

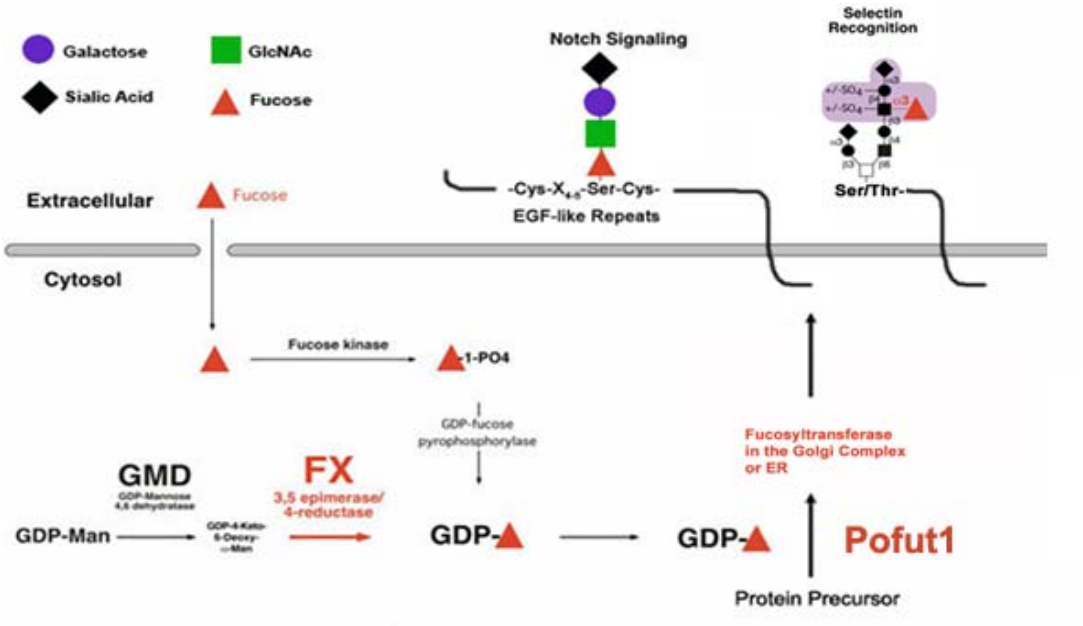


Figure 1-1. *De novo* and *salvage* biosynthesis of *O*-fucose moieties. GDP-fucose is synthesized from GDP-mannose by enzymes encoded by two genes, GMD and FX in the cytoplasm *de novo*. GDP-fucose is transported via the GDP-fucose transporters into the endoplasmic reticulum and the Golgi apparatus where they are incorporated into glycoproteins by fucosyltransferases. In addition, GDP-fucose can be synthesized from free fucose molecules via the salvage pathway. (Adapted from Dr. John B. Lowe)

Figure 1-2

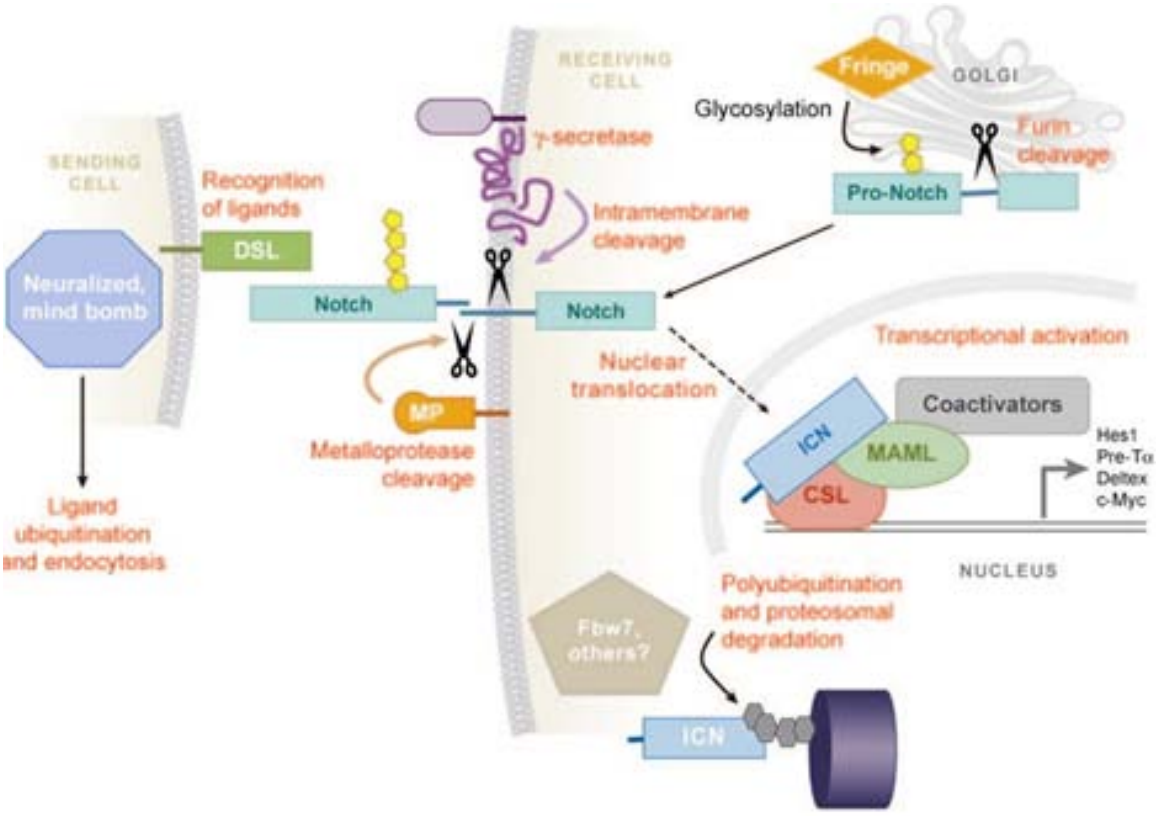


Figure 1-2. Canonical Notch signaling pathway. Post-translational modifications of Notch receptors start with *O*-fucosylation in the endoplasmic reticulum, followed by Fringe glycosylation and Furin cleavage (S1) in the Golgi apparatus to form the hetero-dimerized surface Notch receptors. The binding of Notch to the surface expressing ligands on the neighboring cells initiate S2 and S3 cleavages by surface bound metalloprotease and γ-secretase, respectively. These proteolytic reactions result in the release of the intracellular domain of Notch receptor into the nucleus where the binding between Notch and CSL transcription factor drives the Notch-specific gene expression. (Adapted from Aster JC, et al., *Annu Rev Pathol Mech Dis.* 2008 (82))

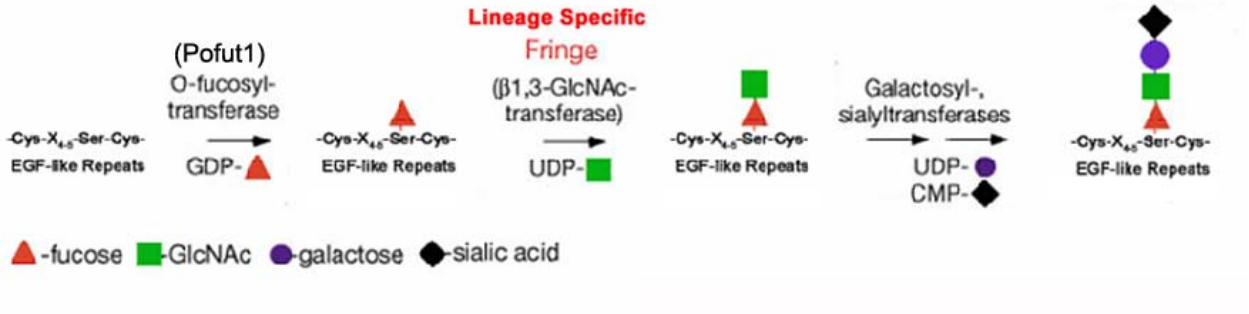


Figure 1-3. Biosynthesis of *O*-fucose moieties on the epidermal growth factor-like (EGF) repeat. Following protein translation, peptides containing the EGF-like repeats with the consensus sequence are modified by Protein O-fucosyltransferase 1 in the endoplasmic reticulum. The *O*-fucosylated EGF-like repeat is transported to the Golgi apparatus where the fucose-containing glycans are further elongated with *N*-acetylglucosamine in the presence of Fringe glycosyltransferase. While in the Golgi apparatus, Fringe-modified *O*-glycans can be extended with galactose and sialyl acid molecules to generate surface expressing or secreted proteins.

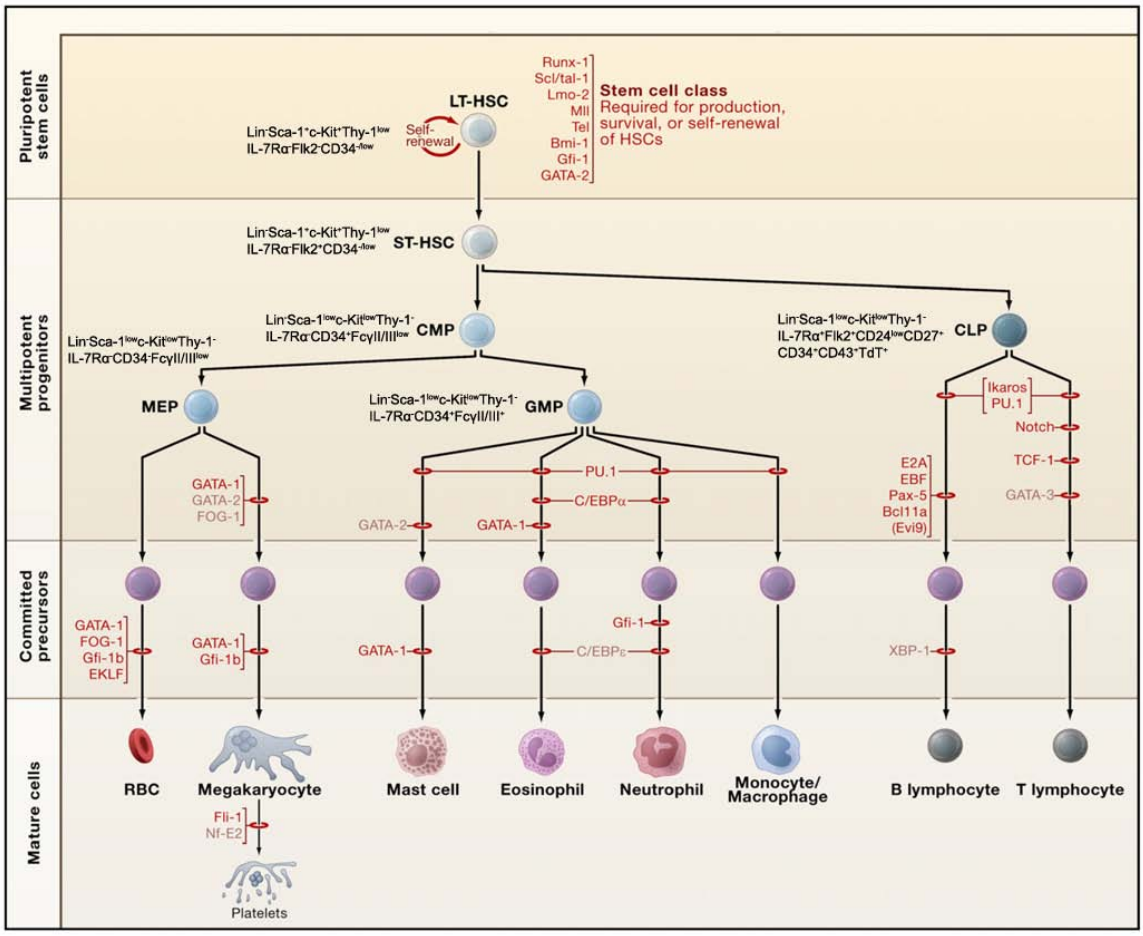


Figure 1-4. Overview of hematopoiesis. Development of the terminally differentiated blood lineage with associated surface markers (in black) and required transcription factors (in red). (Adapted from Orkin SH and Zon LI, *Cell* 2008 (63))

Figure 1-5

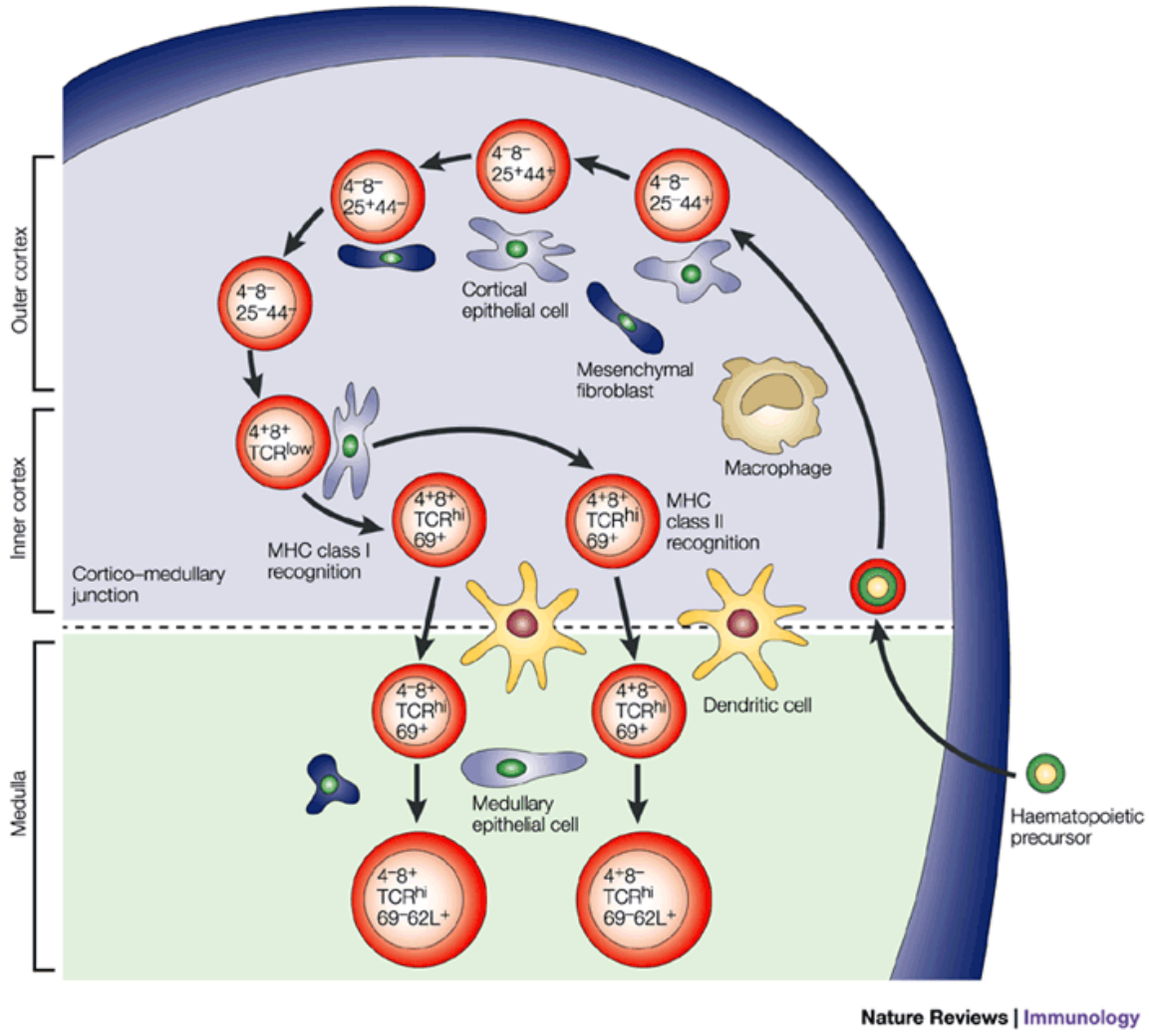


Figure 1-5. Development of T lymphocytes in the thymus. Upon entering the thymus via the cortico-medullary junction, thymic progenitors undergo phenotypic changes as they move from outer cortex towards medulla. Based on the expression levels of TCR, CD69, CD4 and CD8 surface markers, the development of thymocytes can be divided into DN (double negative) and DP (double positive) stages. DN stage can be further subcategorized into DN1 to DN4 based on the expressions of CD44 and CD25 markers. Following selections imposed by the major histocompatibility complex molecules, DP thymocytes mature into L-selectin expressing (CD62L) $CD4^+$ helper or $CD8^+$ cytotoxic single positive T cells. (Adapted from Anderson G and Jenkinson EJ, *Nat Rev Immuno*. 2001 (83))

Table 1-1

Gene	Enzyme	Functions
Fut1	H blood group α2fucosyltransferase	Synthesis of the H antigen in red blood cells
Fut2	Secretor (Se) blood group α2fucosyltransferase	Synthesis of the H atnigen in epithelial tissues and salivary glands
Fut3	Lewis blood group fucosyltransferase	Synthesis of the Lewis^{b} antigen that may contribute to the development of *H. Pylori*
Fut4	Fuc-TIV α3fucosyltransferase	Synthesis of Sialyl Lewis^{X} structure that is essential for leukocyte trafficking
Fut5	Fuc-TV α3fucosyltransferase	
Fut6	Fuc-TVI α3fucosyltransferase	Similar to Fut4
Fut7	Fuc-TVII α3fucosyltransferase	Similar to Fut4
Fut8	Fuc-TVIII α6fucosyltransferase	Expression of this gene may contribute to the malignancy of cancer cells
Fut9	Fuc-TIX α3fucosyltransferase	Production of the Lewis^{X} epitope that may be critical for cell-cell interaction in early embryos
Fut10	Fuc-TX putative α3fucosyltransferase	Enzymatic activities have been identified although functions remain unclear (ref)
Fut11	Fuc-TXI putative α3fucosyltransferase	
Pofut1	Polypeptide *O*-fucosyltransferase 1	Synthesis of *O*-fucose moieties on the EGF-like motifs (pertinent in our study)
Pofut2	Polypeptide *O*-fucosyltransferase 2	Synthesis of *O*-fucose moieties on the thrombospondin 1-like motifs that maybe critical for the protein trafficking

Table 1-1. Known fucosyltransferases and their corresponding functions to date. (Adapted from Becker DJ and Lowe JB, Glycobiology. 2003 (84))

CHAPTER II

O-Fucose-dependent Notch Signaling Regulates Blood Lineage Specification

Abstract

Adult mice carrying a targeted deficiency in the FX locus allele display many hematologic phenotypes, including severe neutrophilia, reduced bone marrow progenitor count, impaired marginal zone B cell growth and faulty mature thymocyte development unless fucose is supplemented in the diet. Our previous analyses have demonstrated that the fucose-dependent defective mature T cell development phenotype in these mice can be partly attributed to faulty Notch activity. While it is evident that Notch modulates T lymphopoiesis, its role in myelopoiesis has yet to be assigned. In this study, we examined the function of fucose-dependent Notch signaling in the development of mature blood cells using mouse embryonic stem cells (ESCs) maintaining targeted deficiency in Pofut1. We first demonstrated that both $CD34^+$ progenitors derived from both Notch1-null and Pofut1-null mouse ESCs failed to give rise to mature T lymphocytes but differentiated into mature myeloid cells while co-culturing with Notch ligand-expressing bone marrow stromal cells *in vitro*. This dysregulation of $CD34^+$ progenitor function may be attributed to suppressed Notch ligand binding and reduced downstream signaling of Notch activity. Consistent with this finding, *in vivo* hematopoietic reconstitution of $CD34^+$ progenitor cells derived from either Notch1-null or Pofut1-null ESCs showed enhanced granulopoiesis with depressed lymphoid lineage development. Moreover, mature thymocytes were undetectable in FX-null mice receiving intra-thymic injections

of Pofut1-null progenitors while rearing on the normal mouse chow. Together, our findings indicate that Notch signaling promotes lymphoid development and suppresses overt myelopoiesis through the control of *O*-fucosylation of Notch receptors.

Introduction

Signal transduction through Notch plays critical roles in both self-renewal and lineage decision in multi-organ and system development. In hematopoiesis, the significance of Notch function in both T-cell lineage differentiation and splenic marginal zone B cells has been documented extensively (85). However, its roles in the decision making between lymphoid and myeloid lineages still remain largely unclear.

Evidence from many studies has demonstrated that Notch signaling is subject to multiple regulating events that modify Notch-Notch ligand interactions or intracellular signaling. As mentioned in Chapter I, *O*-fucose attached to the epidermal growth factor (EGF)-like repeats of Notch receptors can modulate Notch signaling. Recently we reported that mice carrying a targeted deficiency in the FX locus developed chronic myeloproliferation (86). Cellular deficiency of fucosylation in these animals promotes bone marrow progenitors to adopt the myeloid fate at the expense of lymphoid specification. These faulty blood development traits can be either completely or partially reversed when FX-deficient progenitors are reconstituted with constitutively active Notch1 molecules *in vivo*. These findings indicate that faulty myeloid development is at least in part a result of uninhibited suppression of myelopoiesis exerted by Notch. Since FX encodes an enzyme thought to be essential for the constitutive pathway of GDP-fucose synthesis, a donor substrate for the expressions of all fucose-containing

glycoconjugates, this study does not rule out the possible roles of other glycoproteins in the fucose-dependent aberrant hematological phenotypes in FX-deficient mice. To circumvent this issue, we decide to examine the functional roles of *O*-fucose moieties in hematopoiesis using murine embryonic stem cells (ESCs) containing targeted deficient Pofut1, the enzyme known to add *O*-fucose to Notch.

Pofut1 was first identified and cloned from Chinese hamster ovary cells (87). Its homologs in *C. elegans*, *Drosophila*, mouse and human have since been identified (88). Expression profile of Pofut1 transcripts in mice indicates its ubiquitous tissue localization, which is consistent with the system wide *O*-fucose modification. Pofut1 catalyzes the additions of *O*-fucose moieties by transferring fucose molecule from GDP-fucose to the hydroxyl group of serine or threonine residues within an epidermal growth factor like-repeat motif. Previous studies in *Drosophila* demonstrated that Pofut1 is essential for Notch-dependent lineage specification during normal development (56). Furthermore, Pofut1 facilitates folding and trafficking of Notch to the cell surface (57). In mouse, germline homozygosity for Pofut1-null allele leads to embryonic lethality with severe defects in the development of several different organs, a phenotype, which is similar to that of embryos deficient in Notch-specific effectors (58). Collectively, Pofut1 is a core component of Notch signaling pathway.

Given the evidence that Pofut1-mediated sugar modification is critical to normal Notch activity, we speculate that *O*-fucose-modulated Notch signaling regulates the myeloid versus lymphoid diversification. To examine this possibility, we subjected murine ESCs-derived $CD34^+$ progenitors to an *in vitro* T cell differentiation assay. We observed that mouse ESCs carrying targeted deficiencies in either Notch1 or Pofut1 allele

failed to produce mature T lymphocytes while maintaining their ability to generate granulocytes. This phenotype can be attributed to suppressed Notch ligand binding and thus reduced downstream signaling of Notch activity. Consistent with this observation, *in vivo* hematopoietic reconstitution of $CD34^+$ stem cells derived from Notch1-null or Pofut1-null mouse ESCs showed enhanced granulopoiesis with depressed lymphoid lineage development. Moreover, neither Notch1-null nor Pofut1-null progenitors were able to give rise to mature thymocytes upon being injected into the thymi of FX-null mice whose cellular fucosylation was abolished prior to the surgery. Our data indicate that Notch signaling maintains homeostasis of hematopoietic lineage determination by promoting lymphoid development and suppressing overt myelopoiesis, in part through processes controlled by *O*-fucosylation of Notch receptors.

Methods

ES cell lines

Notch1-null ES cell line was kindly provided by Dr. Raphael Kopan (Washington University School of Medicine). Pofut1-null ES cells were kindly provided by Dr. Pamela Stanley (Albert Einstein College of Medicine). The Pofut2-null mouse ES line was generated from blastocyst outgrowths of Pofut2 heterozygotes crossed at the Case Transgenic & Targeting Facility (BayGenomics, ES cell line RST434). The genotype was then confirmed by PCR.

Antibodies

Antibodies used for cell surface marker characterization include: B220, CD21, CD23, CD4, CD8, CD11b, Gr-1 and CD34.

Flow cytometric analysis and cell sorting

Flow cytometric analysis was performed using FACS Aria (Becton Dickinson, Franklin Lakes, NJ). For analysis of surface marker expression, all samples were stained with fluorophore-conjugated antibodies in Hanks' Balanced Salt Solution (HBSS) supplemented with 0.5% BSA at 4^{o}C for 20 minutes. Appropriate isotype controls were included. Data analysis was completed using FACSDiva software (Becton Dickinson). When isolating multipotent progenitor cells (MPP) ($Lin^{-}Sca1^{+}c\text{-}kit^{+}Flt3^{+}$), lineage-depleted cells were further stained with streptavidin-APC-Cy7, fluorescein isothiocyante (FITC)-anti-Sca-1, phycoerythrin (PE)-anti-Flt3, and allophycocyanin (APC)-anti-c-kit, and sorted using FACSAria.

ES cell *in vitro* hematopoietic differentiation

ES cells (8×10^{3}) were cultured on Iscove's modified Dulbecco's medium containing 1% MC (M03120, StemCell Technologies) and 40 ng/ml stem cell factor (SCF) (R&D Systems, Minneapolis, MN). After 7-10 days, the embryoid bodies were removed, and $CD34^{+}$ cells were isolated by biotinylated rat anti-mouse CD34 and anti-biotin-beads (Miltenyi, Auburn, CA). These cells were then seeded onto OP9-control or OP9-Notch ligand expressing bone marrow stromal cells. The co-culture was maintained in RPMI medium supplemented with 10% of fetal bovine serum, 5 ng/ml of mFlt3 ligand and 5 ng/ml of IL-7. Cells were fed every three days and transferred to fresh bone marrow stromal cell culture every six days. Cells were recovered from the day 15 culture and analyzed by FITC-anti-CD11b or CD25; PE-anti-Ter119 or CD44; APC-anti-B220 or CD4; APC-Cy7-anti-CD8; and PE-Cy7-anti-CD45 (BD Biosciences, San Jose, CA). Cell surface expression of Notch receptors in $CD34^{+}$ HPCs was characterized by staining

cells with PE-conjugated anti-Notch1, Notch2, Notch3 and Notch4 antibodies (Biolegend, San Diego, CA).

***in vivo* assessment of ESC-derived hematopoietic development by intra-femoral transplantation and intra-thymic transplantation**

ESCs were induced to differentiate toward hematopoiesis as described above. A single cell suspension of $CD34^+$ cells (10^5 cells/20 μl) was directly injected into femurs of recipients (Ly5.1) that were sub-lethally (5.5 Gy) or lethally irradiated (9.5 Gy) 24h prior to the transplantation (167). The presence of donor derived (ESC-Ly5.2) T, B and granulocytes was determined by flow analysis of peripheral blood collected 2, 4, 8, 12 wks, and monthly after injection. Of 74 mice injected, only one mouse developed teratoma. No mice showed signs of weight loss or hunched back which are suggestive of graft-versus-host-disease. For intra-thymic transplantation, $CD34^+$ cells (10^5 cells/20 μl) were injected into thymic lobes of anesthetized $FX^{-/-}$ mice that were reared on fucose-supplemented chow until eight wks of age and then maintained on standard chow for 10 days prior to the transplantation. T lymphopoiesis was analyzed by flow cytometry on total thymocytes collected two weeks after the surgery.

Recombinant mouse Notch ligand binding assays

Mouse Dll1, Dll4 and J1 cDNAs were generated by RT-PCR using total RNA isolated from a new born mouse thymus. The nucleotides encoding the extracellular domain of each ligand was fused in frame with the Fc region of human IgG1 in pCDNA1 vector. HEK 293T cells were transiently transfected with these constructs using lipofectamine 2000. After 24 hours, cells were harvested and replated at a 1:10 dilution in serum free medium (Hyclone) supplemented with 1% FBS. Ten days following the

replating, supernatant was collected and concentrated using Centricon-Plus 70 filter units with a membrane size of 30 KDa (Millipore). The concentration of each soluble Notch ligand was approximated using enzyme-linked immunoabsorbent assays (ELISA). The optical density values were converted into concentrations using the standard curve of recombinant human CD14 from Sigma Aldrich. Expression of these soluble ligands was further visualized by Western blot using horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc). J2 soluble ligand was collected from the supernatant of a stable J2 cell line culture (a kind gift of Dr. Conrad Bleul, Freiburg, Germany). Binding assay was performed by incubating ES cells with recombinant Notch ligand in Hanks balanced salt solutions supplemented with 1 mM of Ca^{2+} for 20 minutes at room temperature. Binding results was obtained by flow analysis using PE-anti-human IgG Fc.

Statistical analysis

Data are presented as means plus or minus SD, unless otherwise stated. Statistical significance was assessed by Student *t*-test.

Results

ES cells deficient in Notch1 or Pofut1 exhibit suppressed lymphopoiesis but enhanced myelopoiesis *in vitro*

Adult mice deficient in global cellular fucosylation give rise to enhanced myelopoiesis and suppressed lymphopoiesis. These phenotypes are partially reversed by constitutively active Notch1 molecules. Our previous observations are consistent with the hypothesis that hematologic consequences of fucose deficiency can be attributed to loss of Notch-dependent control on lineage homeostasis as a consequence of loss of Notch *O*-

fucosylation. To substantiate this notion, we sought to assess the role of Protein *O*-fucosyltransferase-1 (Pofut1) in both myelopoiesis and lymphopoiesis. Pofut1 is essential in catalyzing the appendage of *O*-fucose to consensus EGF repeats on the extracellular domains of Notch receptors. Germ line deficiency of Pofut1 and Notch1 are embryonic lethal, which prohibit the use of their hematopoietic progenitors to study the roles of Notch1 and Pofut1 in myelopoiesis (58, 89). To determine if the aberrant myeloid reconstitution by $FX^{-/-}$ cells is due specifically to suppressed Notch signaling as a consequence of loss of Notch1 receptor or *O*-fucose moieties on Notch receptors, we analyzed the blood lineage specification from mouse embryonic stem cells (ESCs) carrying either a Notch1-null or Pofut1-null allele using a modified *in vitro* culture system (Figure 2-1). ESCs deficient in Pofut2, which catalyzes the additions of *O*-fucose to thrombospondin type 1 repeats (90), a protein motif that is not present on Notch receptors or their ligands, were studied in parallel as controls. Here, ESCs were initially differentiated into $CD34^+$ cells in methylcellulose prior to co-culturing with OP9 cells bearing different Notch ligands (Figure 2-1). (91)

Following a 15-day co-culture with control OP9 cells, WT ESC-derived $CD34^+$ cells gave rise to myeloid cells (Figure 2-2) and B lymphoid cells. In contrast, these cells differentiated into $CD8^+$ single-positive (SP) or $CD4^+/CD8^+$ double-positive (DP) mature T cells when co-cultured with OP9s bearing Jagged2, Dll1, or Dll4. The induction of T lymphopoiesis facilitated by Notch-ligand-expressing OP9s is consistent with previously reported observations. The development of mature T cells in these cultures positively correlates with the up-regulation of Notch-specific transcripts, including Hes1 and Deltex1, as well as efficient soluble Notch ligand bindings (Figure 2-3). $CD34^+$

progenitors derived from Pofut2$^{-/-}$ ESCs also showed comparable T lymphopoiesis to WT cells under the same culturing condition. Furthermore, these cells exhibited similar levels of Notch activation and Notch ligand staining when compared with WT control.

Progenitors derived from Notch1$^{-/-}$ESCs failed to generate T cells in the presence of Notch-ligand. Instead, the vast majority of cells expressed the Gr-1 surface marker, which accounted for between 39.4% and 45.3% of the total hematopoietic cells in the culture (Figure 2-2). Absence of mature T cells in these cultures correlated with the basal levels of Hes1 and Deltex1 expression and the background level of Notch ligand staining, all of which indicated the abrogation of Notch activation (Figure 2-3). Regardless of whether Notch ligand was present in the culture, the vast majority of cells derived from Pofut1$^{-/-}$ CD34^{+} progenitors were myeloid cells while mature T cells were absent. This cellular distribution was similar to that of Notch1$^{-/-}$ culture. Moreover, expressions of Notch targeted transcripts and ligand binding were markedly decreased in Pofut1$^{-/-}$ progenitors although not absent. These findings suggested that the partial suppression of Notch activity in the absence of *O*-fucosylation on Notch yields hematologic phenotypes similar to those observed in Notch1$^{-/-}$ cells. Reduced soluble ligand staining in the absence of *O*-fucose molecules did not, however, account for the possibility that Notch receptors without *O*-fucosylation are not trafficked properly to the cell surface. Immunophenotyping of Notch expression using antibodies specific to an individual homolog revealed that Notch1 was absent in Notch1$^{-/-}$ and mildly reduced in Pofut1$^{-/-}$ progenitors. Expressions of other Notch receptors in Pofut1$^{-/-}$ progenitors were not up-regulated when compared to those in Notch1$^{-/-}$ cells (Figure 2-3). Together, these findings

suggest that the defective Notch-ligand binding mediated via Notch1 is the primary cause for the phenotype seen in Pofut1$^{-/-}$ cells.

Aberrant *in vivo* hematopoiesis from ES cells deficient in Notch1 receptor or Notch *O*-fucose modification

To confirm that the anomalous lineage development of Notch1$^{-/-}$ and Pofut1$^{-/-}$ progenitors *in vitro* is reflective of their *in vivo* features, we injected ESC-derived CD34^{+}; Ly5.2^{+} hematopoietic progenitors (HPCs) into non-lethally irradiated recipient mice (Ly5.1^{+}). Figure 2-4 illustrates the experimental approach of intra-femoral administrations. 1×10^5 CD34^{+} cells were directly injected into a femur of the recipients (Ly5.1). The earliest hematopoietic reconstitution from ESC-derived HPCs was observed 2 wks after the injection of CD34^{+} cells. By 12 wks, the proportion of ESC-derived Ly5.2^{+} cells in the peripheral blood ranged from 52 ± 25% to 79 ± 13% for all cells transplanted. The fraction of Ly5.2^{+} cells remained steady through 16~20 wks after the transplantation. Flow cytometric analysis of peripheral blood revealed that Ly5.2^{+} population was comprised of 25.1 ± 8.4%, 54.1 ± 9.2% and 12.3 ± 1.4% of WT HPC-derived T cells, B cells and granulocytes, respectively. A similar lineage distribution was found in mice transplanted with Pofut2$^{-/-}$ HPCs (Figure 2-5). At 12 wks, mature T and B cells derived from Notch1$^{-/-}$ HPCs represented only 8.8 ± 2.5% and 16.9 ± 3.3% of the transplanted cells, respectively. In comparison, the distribution of granulocytes from Notch1$^{-/-}$ HPCs was 57.7 ± 9.6%. The lineage distribution of Pofut1$^{-/-}$ HPC donors had decreased T and B lymphoid compartments and increased granulocytic chimerism (Figure 2-5) and neutrophilia. As summarized in Table 2-1, 12 weeks following

transplantation, Notch1$^{-/-}$ and Pofut1$^{-/-}$ HPCs yielded a lymphoid-suppressed but myeloid-expanded phenotype. The absolute count of B cells derived from donors carrying Notch1$^{-/-}$ or Pofut1$^{-/-}$ alleles remained similar due to increased WBC numbers. The proportion and absolute numbers of T cells, B cells and granulocytes generated from hosts (Ly5.1^{+}) were similar among mice receiving all four ESC lines, suggesting that the lymphoid-suppressive and myeloid-enhancing phenotype in recipients transplanted with either Notch1$^{-/-}$ or Pofut1$^{-/-}$ HPC is cell-autonomous.

A decreased T lymphocyte population in the periphery of individuals receiving Notch1$^{-/-}$ or Pofut1$^{-/-}$ HPCs was due to suppressed thymic T lymphopoiesis. In one representative experiment, absolute counts of donor-derived thymocytes (Ly5.2^{+}) were 0.3×10^6 (Notch1$^{-/-}$) and 0.04×10^6 (Pofut1$^{-/-}$), whereas donor thymocyte numbers in mice receiving WT or Pofut2$^{-/-}$ HPCs were 34.4×10^6 and 29.1×10^6, respectively. Both single positive (SP) and double positive (DP) T cells derived from either Notch1$^{-/-}$ or Pofut1$^{-/-}$ HPCs were significantly decreased (Figure 2-5). In comparison, the development of mature thymocytes generated from the hosts (Ly5.1^{+}) was not disturbed.

Faulty thymic T lymphopoiesis from ES cells deficient in Notch1 or Pofut1

The site of T lymphopoiesis occurs in the thymus. The key molecular events to successful development of mature T thymocytes have been characterized extensively. Upon their release from the bone marrow, early thymic progenitors circulate in the periphery where adhesion molecules critical to thymic homing are acquired. Numerous fucosylated surface glycoproteins have been implicated in lymphoid progenitor homing to thymus (66, 92). To exclude the possibility that faulty thymopoiesis in mice receiving

intra-femoral administrations of Notch1- or Pofut1-deficient $CD34^{+}$ cells is due to homing defect, we injected these progenitors directly into the thymus of fucose-depleted $FX^{-/-}$ mice (Ly5.1) whose ability to generate the host thymocyte is compromised (Figure 2-6). Two weeks following the surgery, single cell suspension was prepared from thymi for the evaluation of T-specific surface markers. Similar to our previous observation, mice receiving WT and $Pofut2^{-/-}$ HPCs produced a robust SP and DP T-cell population while thymi from those injected with HPCs bearing deficient Notch1 or Pofut1 were severely hypoplastic without mature thymocyte detected (Figure 2-7).

Discussion

In this study, we confirmed our previous finding that *O*-fucose moieties on Notch play an essential role in regulating blood lineage determination. Using the murine embryonic stem cell (ESC) lines, we demonstrated that insufficient *O*-fucosylation of Notch receptors leads to enhanced myeloid lineage development at the expense of mature lymphoid differentiation.

To elucidate the role of *O*-fucose moieties in hematopoietic lineage diversification, we utilized the *in vitro* Notch-ligand-mediated culture system to generate mature T cells from embryonic stem cells that was previously devised by Zúñiga-Pflücker and colleagues (93). The foremost challenge of utilizing this assay is its consistency in generating a robust population of mature T lymphocytes. To circumvent this difficulty, we modified the original assay by simply differentiating ESCs into $CD34^{+}$ progenitors in methylcellulose prior to subjecting these cells to the T cell development

assay. The addition of this initial culturing step allowed us to reliably obtain mature T cells from ESCs *in vitro*.

Using this modified assay, our data show that WT ESCs give rise to a balanced leukocyte lineage development whereas loss of Notch1 results in a diminished lymphoid and enhanced myeloid lineage. Our findings suggest that the lymphoid versus myeloid lineage diversification is exerted by Notch activity in an *O*-fucose dependent manner. Complement to this observation, mice reconstituted with Notch1$^{-/-}$ or Pofut1$^{-/-}$ progenitors had an enhanced myeloid and an inhibited lymphoid development. This finding is consistent with those observed in the FX$^{-/-}$ mice, suggesting that the aberrant lineage specification phenotype observed in FX$^{-/-}$ mice can be largely attributed to the abnormal Notch signaling control of hematopoiesis in the absence of *O*-fucose moieties. Together, these findings strongly support the notion that Notch signaling is essential for blood lineage homeostasis by promoting lymphoid development while suppressing overt myelopoiesis.

O-fucose is a relatively rare entity whose existence is detected on a handful of cell surface and secreted proteins that are composed of EGF-like repeat motifs, including Cripto and coagulation factors (94-96). These glycoconjugates are also attached to proteins containing thrombospondin-like repeat (TSR) domains. Additions of *O*-fucose to TSR repeats are catalyzed by Protein *O*-fucosyltransferase 2. Using the same *in vitro* culturing system and the intra-femoral injection assay, ESCs maintaining targeted deletion in the Pofut2 allele appeared to preserve the ability to undergo normal blood lineage differentiation. This finding does not only suggest that Pofut2 is not essential for

the development of mature blood cells, but also to offer further evidence that *O*-fucose glycans are critical to hematopoiesis only if they are present on Notch.

Cellular mechanisms by which *O*-fucose moieties modulate Notch activity and blood cell lineage are likely via the regulation of interactions between Notch and its ligands. Soluble Notch ligand staining of Pofut1$^{-/-}$ ESC-derived progenitors is markedly reduced. The faulty ligand-receptor interaction crippled the ability of hematopoietic progenitors to transcribe Notch-specific targets. This reduced ligand-receptor binding phenotype may also stem from the decreased cell surface expression of Notch in Pofut1$^{-/-}$ HPCs. This is consistent with the observations made by Stahl and colleagues, whose study indicate that Pofut1$^{-/-}$ ES cells exhibit suppressed Notch signaling (97).

Commitment of hematopoietic progenitors to either the myeloid or lymphoid lineage is regulated by the activation of several key transcription factors. Up-regulation of target transcripts yields a gene expression profile characteristic of that specific lineage (98). While receptor tyrosine kinase Flt3 and Notch1 are important for the initiation of lymphoid lineage (99), PU.1 and C/EBPα are essential for the commitment of myeloid lineage (100). It is known that Notch promotes T lymphopoiesis by stimulating T cell-specific transcription factors while down- regulating myeloid transcription factors (98, 101). Consistent with this notion, our data illustrate that the expressions of lymphoid/myeloid lineage-specific transcripts are modulated by *O*-fucose dependent Notch activity as murine progenitors derived from Pofut1-deficient ESCs had markedly reduced response to Notch-mediated T lineage specification. We speculate that this weak Notch activity is a result of diminished interactions between Notch and its ligands in the

absence of *O*-fucose moieties. It remains unclear how Notch suppresses the expression of myeloid-specific transcripts in lymphoid-primed progenitors.

Notch1, Notch2 and Notch3 are expressed in HSCs, each of which has consensus sequences known to be modified by *O*-fucose. The similar faulty hematopoietic reconstitution phenotypes between Notch1$^{-/-}$ and Pofut1$^{-/-}$ progenitors suggest that among members of the Notch family, Notch1 may be the primary player in blood lineage maintenance. This notion is supported by our findings that expression of constitutively active Notch1 partially suppressed myeloproliferation conferred by the FX$^{-/-}$ marrow progenitors (86). However, we cannot exclude the possibility that the suppression of myeloproliferation in the bone marrow is secondary to the Notch1-mediated expansion of immature T cells. For instance, Notch2 has been shown to support the differentiation of mast cells from common myeloid progenitors (102). Our observation that Notch1 and Pofut1 deficiency lead to enhanced myelopoiesis is not reported in a previously report where conditional inactivation of Notch1 inhibits T cell development without the disturbance of other lineages (44). This discrepancy may be due to the redundant functions of other Notch receptors in myelopoietic suppression. Alternatively, the enhanced myeloid reconstitution of Notch1-deficient CD34^{+} progenitors may reflect a developmental stage-specific effect of ES-derived progenitors on hematopoiesis.

Aside from suppressed T lymphopoiesis and enhanced myelopoiesis in mice receiving progenitors derived from Notch1- and Pofut1-deficient ESCs, these animals appeared to have a normal peripheral B lymphoid compartment. Despite the normal B-cell compartment in the periphery, these mice had reduced T1 B-cells and marginal zone B-cells. The changes in the B cell compartment including its subsets were also observed

in $FX^{-/-}$ mice while rearing on the normal mouse chow, suggesting a role of Notch in the development of splenic B-cells. Since Notch2 has been previously implicated in the formation of marginal zone B-cell population (103, 104), our finding elucidates an essential role of *O*-fucose on Notch2 in specific B-cell subsets.

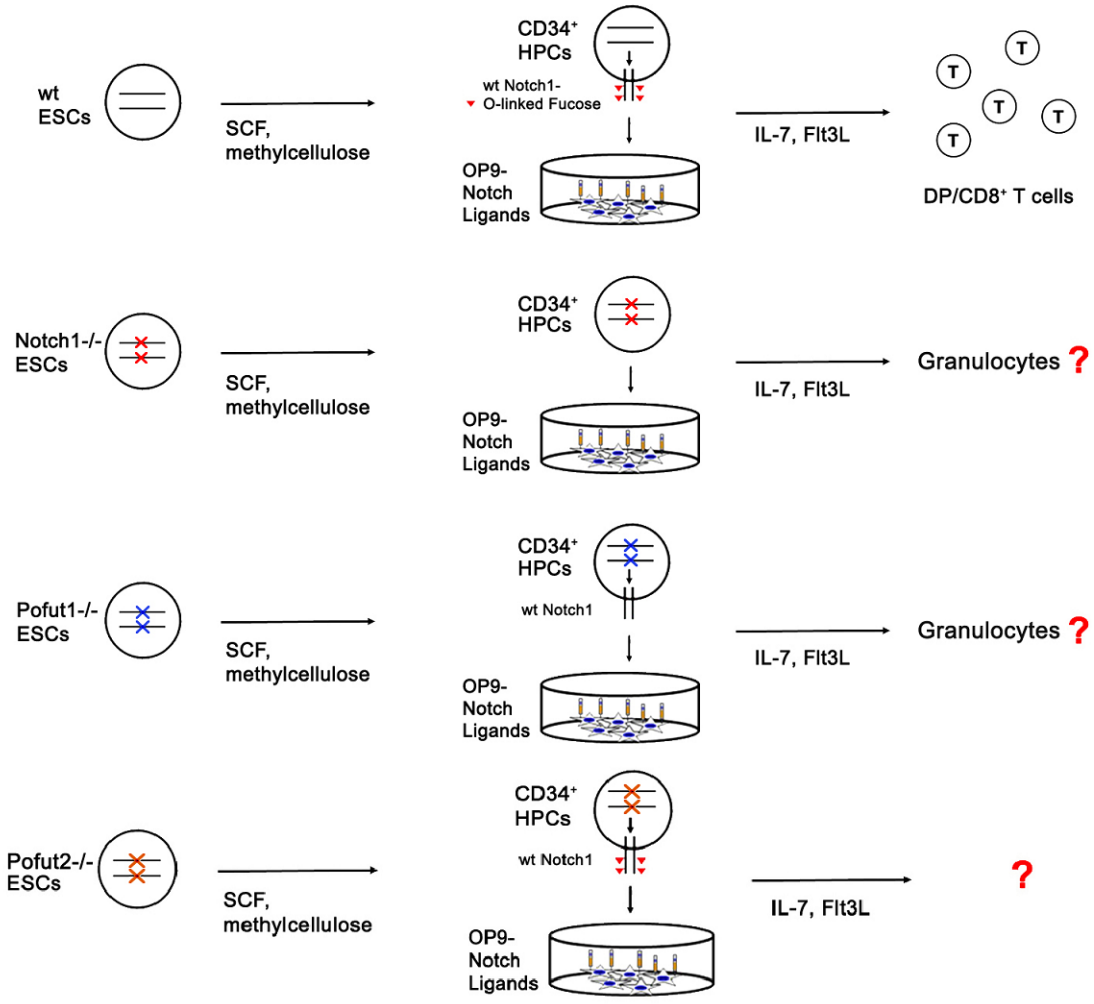
In this chapter, I described our approach to elucidate the role of complex carbohydrate in lineage determination. Using various mouse embryonic stem cell lines in both *in vitro* and *in vivo* assays, we demonstrated that *O*-fucose moieties on Notch receptors positively facilitate the T lymphopoiesis from hematopoietic progenitors at the expense of myelopoiesis. A series of questions still remain unanswered, however. For instance, can we recapitulate the aberrant hematological phenotypes in mice that are genetically engineered to be conditionally defective in the *O*-fucose synthesis? Furthermore, are these genetic traits regulated cell-autonomous events, environmental factors or both? In the following chapter, I will present evidence in an attempt to answer some of these questions and hope to establish a role for *O*-fucose moieties in the cellular response to hematopoietic lineage specification signals.

Notes to Chapter II

I want to first thank Quanjian Yan and Lebing Wei for setting up the quantitative real-time polymerase chain reactions and performing the intra-femoral injections. I also want to thank Dr. David LePage at the Case Transgenic & Targeting Facility for the generation of Pofut2-deficient mouse embryonic stem cell line. I also want to thank Dr. Lan Zhou for her suggestions regarding the OP9 co-cultures. The constructs containing soluble Notch ligands were made by Bronislawa Petryniak.

Figure 2-1. Strategy for generating mature T cells from murine embryonic stem cells (ESCs) *in vitro*. ESCs were first cultured in the methylcellulose gel supplemented with stem cell factor. Progenitors were then positively selected for the expression of CD34 surface marker. These cells were subjected to co-culture with OP9 bone marrow stromal cells expressing Notch ligands in the presence of IL-7 and Flt3L. 21 days following the culture, cells were then harvested for the analysis of mature B-, T-cell and granulocyte surface expression.

Figure 2-1


wt
ESCs
SCF,
methylcellulose
CD34+
HPCs
wt Notch1-
O-linked Fucose
OP9-
Notch
Ligands
IL-7, Flt3L
T
DP/CD8+ T cells
Notch1-/-
ESCs
Granulocytes ?
Pofut1-/-
ESCs
wt Notch1
Pofut2-/-
ESCs
?

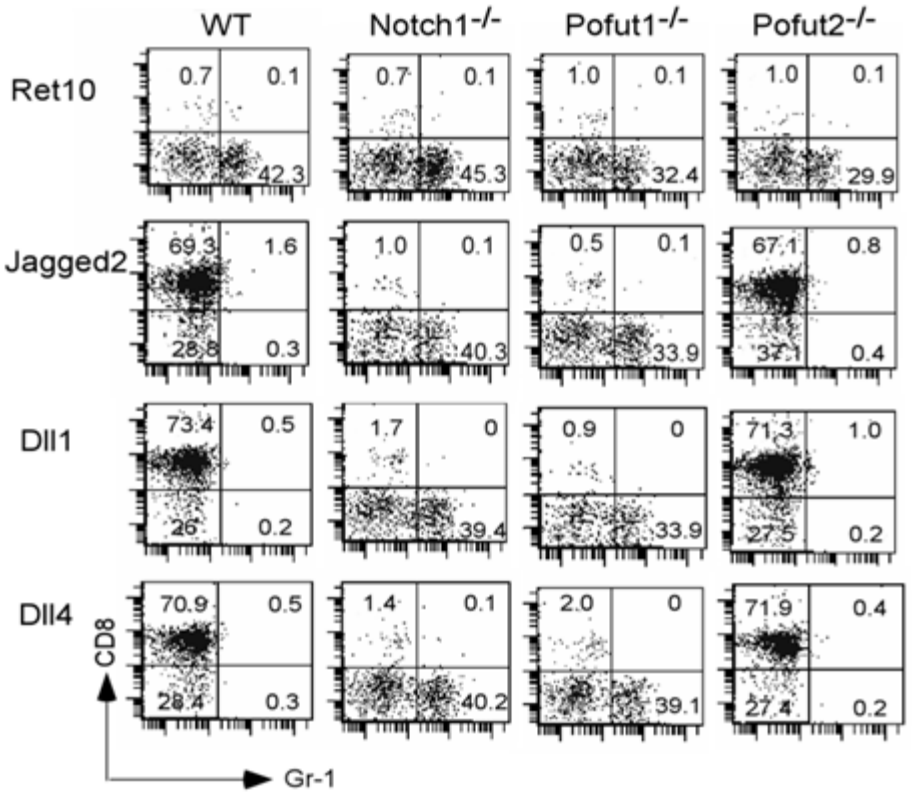
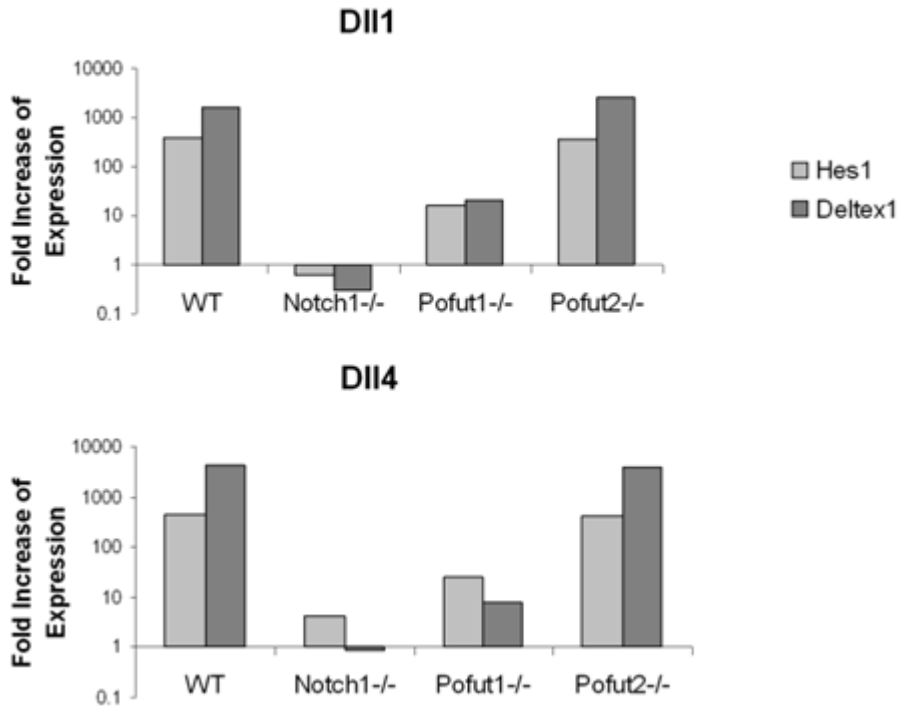


Figure 2-2. Surface expression of CD8 and Gr-1 from *in vitro* T cell differentiation assay. Progenitors derived from WT, Notch1-/-, Pofut1-/- or Pofut2-/- murine embryonic stem cells were co-cultured with control OP9 cells (Ret10) or OP9 bearing one of the Notch ligands, Jagged2, Dll1 or Dll4. The percentage of CD8+ and Gr-1+ cell population is denoted in the corresponding quadrant.

Figure 2-3. Down-regulated expressions of Notch-specific transcripts in the absence of Pofut1. a. Relative expression levels of Hes1 and Deltex1 in WT, Notch1$^{-/-}$, Pofut1$^{-/-}$ or Pofut2$^{-/-}$ ESC-derived progenitors in response to Dll1 and Dll4 induction. The transcript fold increase was normalized to that detected in the control culture, whose fold increases was set to 1. Data are representative of three independently performed experiments. b. Flow cytometric analysis for the bindings of soluble Dll1 and Dll4 to progenitors derived from murine ESCs. Binding strength is expressed as MFI of the soluble ligand staining. Human IgG (hIgG FC) was used as negative control for the measurement of non-specific binding. MFI: mean fluorescence intensity.

Figure 2-3

a.


Dll1
Fold Increase of Expression
10000
1000
100
10
1
0.1
WT
Notch1-/-
Pofut1-/-
Pofut2-/-
Hes1
Deltex1
Dll4
Fold Increase of Expression
10000
1000
100
10
1
0.1
WT
Notch1-/-
Pofut1-/-
Pofut2-/-

b.

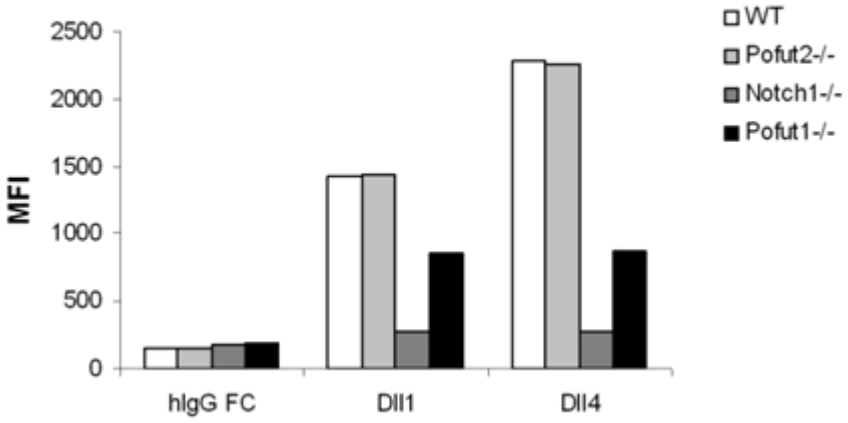

MFI
2500
2000
1500
1000
500
0
hIgG FC
Dll1
Dll4
WT
Pofut2-/-
Notch1-/-
Pofut1-/-

Figure 2-4

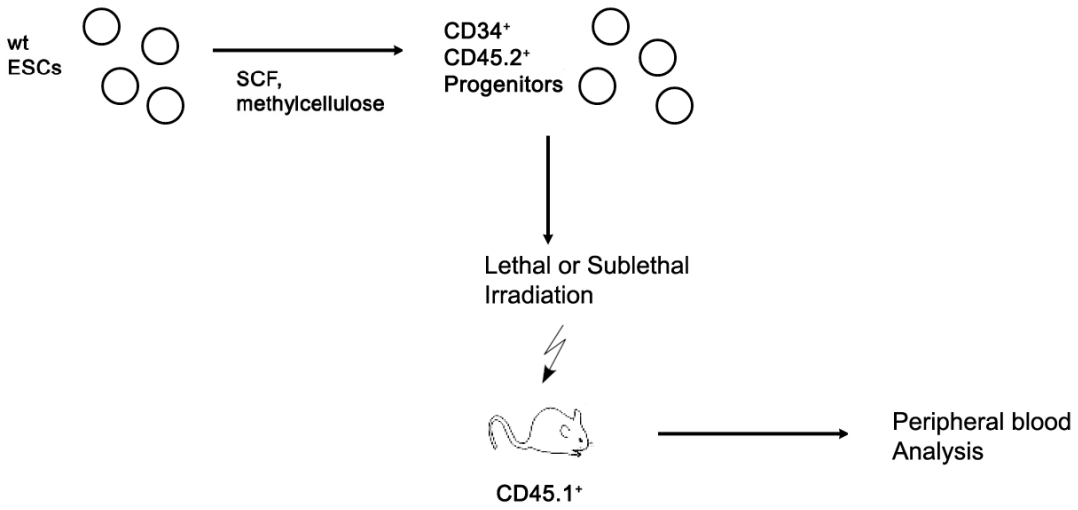


Figure 2-4. Schematic diagram depicting direct intra-femoral injections of ESC-derived progenitors. ESCs were differentiated into $CD34^+$ progenitors prior to being directly administered intra-femorally into recipient mice lethally or sub-lethally irradiated. Peripheral blood analysis was performed at indicated time following the transplant using flow cytometry.

Figure 2-5. Distribution of mature blood cell population in the periphery after intra-femoral transplantation. Percentage of CD4/8^{+}, B220^{+} and Gr-1^{+} population derived from intra-femorally injected progenitors two, four, eight and 12 weeks after the transplantation. N=4 for each embryonic stem cell line. Error bars represent the standard deviation.

Figure 2-5

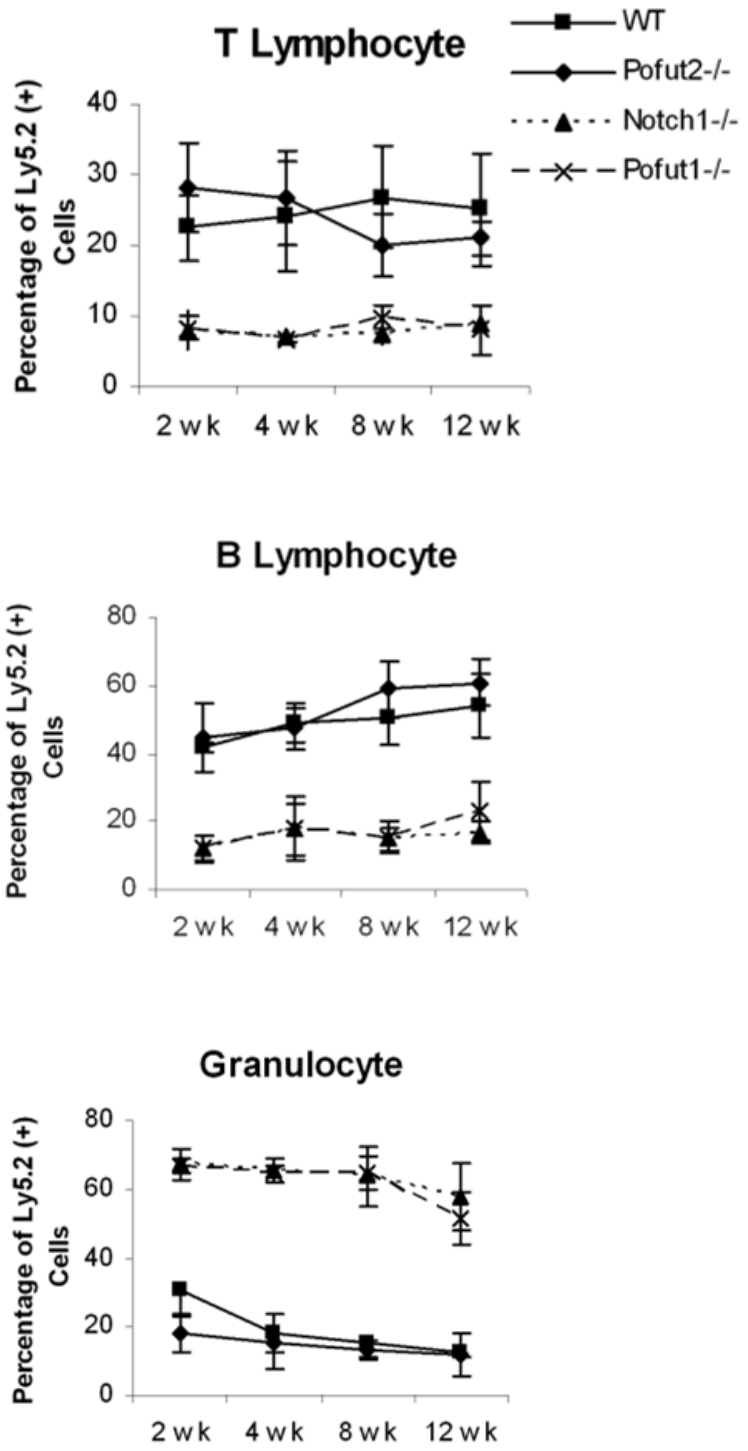

T Lymphocyte
WT
Pofut2-/-
Notch1-/-
Pofut1-/-
Percentage of Ly5.2 (+) Cells
40
30
20
10
0
2 wk
4 wk
8 wk
12 wk
B Lymphocyte
Percentage of Ly5.2 (+) Cells
80
60
40
20
0
2 wk
4 wk
8 wk
12 wk
Granulocyte
Percentage of Ly5.2 (+) Cells
80
60
40
20
0
2 wk
4 wk
8 wk
12 wk

Figure 2-6

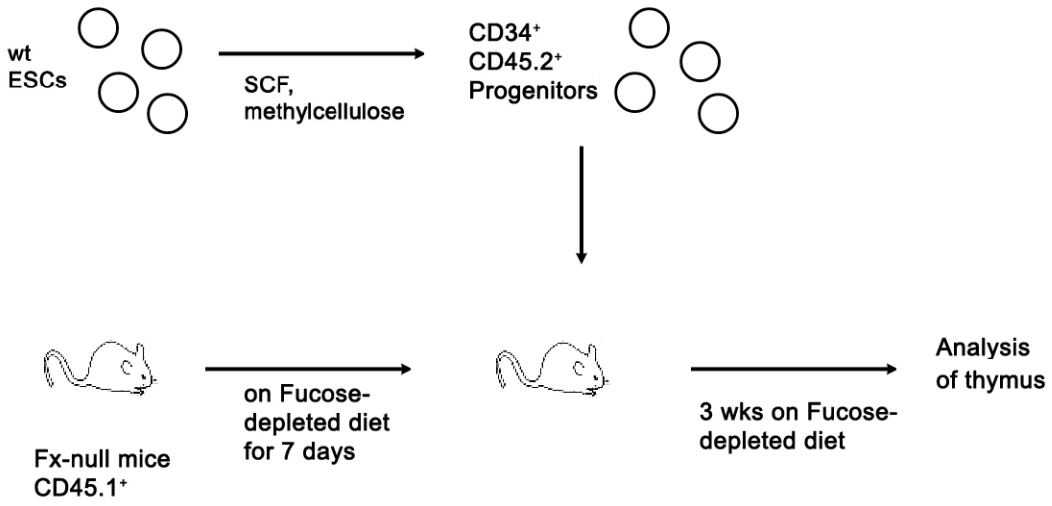


Figure 2-6. Illustration presenting our approach to intra-thymic injections of ESC-derived progenitors. $CD34^{+}$ progenitors derived from ESCs were injected intra-thymically into recipient $FX^{-/-}$ mice rearing on the normal mouse chow for seven days. Following the injection, recipients continued to receive normal chow for three weeks. Thymocytes were then isolated for analysis.

Figure 2-7. Thymocytes differentiated from progenitors that were intra-thymically injected into FX$^{-/-}$ mice. Surface expressions of CD4 and CD8 were examined in thymocytes derived from WT, Notch1$^{-/-}$, Pofut1$^{-/-}$ and Pofut2$^{-/-}$ donor progenitors (CD45.2^{+}). Data are representation of five individual mice. Number and percentage of each cell population is denoted in the appropriate quadrant (per 10,000 events).

Figure 2-7

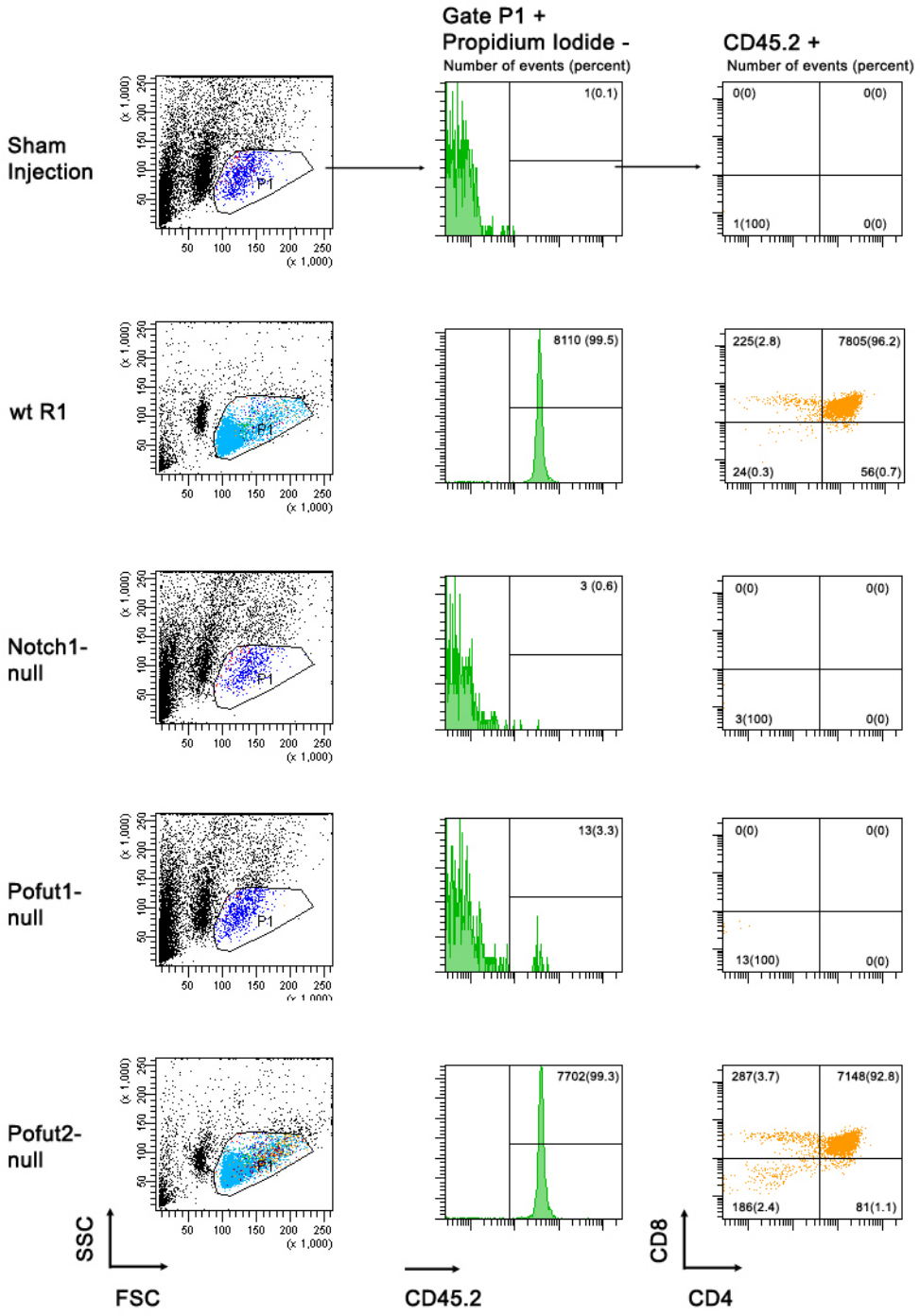

Gate P1 +
Propidium Iodide -
Number of events (percent)
CD45.2 +
Number of events (percent)
Sham Injection
wt R1
Notch1-null
Pofut1-null
Pofut2-null
P1
1(0.1)
0(0)
0(0)
1(100)
0(0)
8110 (99.5)
225(2.8)
7805(96.2)
24(0.3)
56(0.7)
3 (0.6)
3(100)
13(3.3)
13(100)
7702(99.3)
287(3.7)
7148(92.8)
186(2.4)
81(1.1)
SSC
FSC
CD45.2
CD8
CD4

Table 2-1

Peripheral Blood						
	Ly5.2+ Cells (n=4)			**Ly5.1+ Cells (n=4)**		
	T (%)	B (%)	G (%)	T (%)	B (%)	G (%)
WT	25.1 ±8.4	54.1 ±9.2	12.3 ±1.4	44.8 ±9.8	25.2 ±9.4	16.7 ±7.0
Pofut2$^{-/-}$	21.0 ±2.5	60.9 ±6.6	11.6 ±6.2	43.5 ±6.5	24.4 ±10.6	23.0 ±12.5
Notch1$^{-/-}$	8.8 ±2.5**	16.9 ±3.2*	57.7 ±9.6**	32.3 ±2.0	34.3 ±3.9	23.8 ±1.5
Pofut1$^{-/-}$	8.0 ±3.4**	23.3 ±8.6*	51.6 ±7.6**	36.7 ±9.4	30.5 ±6.8	17.2 ±7.2

Table 2-1: Lineage distributions of donor-contributed Ly5.2 and host-derived Ly5.1 cells. The percentage of cell population was obtained 12 weeks following the intra-femoral transplantation of ESC-derived progenitors in non-lethally irradiated recipient mice. Data are presented as mean ± standard deviation. T: T-cell; B: B-cell; G: Granulocyte

CHAPTER III

Aberrant Notch signaling and Hematopoietic Homeostasis in Mice with Conditional Deficiency of Protein of Fucosyltransferase 1 (Pofut1)

Abstract

In this study, we continue in the quest to address the physiologic function of *O*-fucose dependent Notch activity in hematopoiesis using a mouse model maintaining a conditional targeted deficiency in the Pofut1 locus. These mice produced hematologic phenotypes that are reminiscent of those found in mice engineered to be completely deficient in global cellular fucosylation, including low hematopoietic progenitor count, neutrophilia, thymic hypoplasia and faulty marginal zone B cell development. Each of these phenotypes was due to loss of cell-autonomously and environmentally modulated Notch signaling. Consistent with this finding, bone marrow progenitors bearing deficient Pofut1 functions fail to generate mature T lymphocytes but differentiate into myeloid cells while co-culturing with Dll4-expressing bone marrow stromal cells *in vitro*. Transplanted Pofut1-deficient marrow progenitors were able to differentiate into mature thymocytes in the wild-type thymic environment although when competing against wild-type cells, these progenitors gave rise to nearly no mature thymocytes. The cellular mechanisms by which *O*- fucose molecules regulate blood cell development are by modulating the cell surface expression of Notch receptors and the interaction between Notch and its ligands. Further, Pofut1 displayed haploinsufficiency in restoring myeloid homeostasis in global fucosylation-deficient mice through exogenous fucose-dependent

salvage pathway. Taken together, our data support an essential role of Pofut1 in Notch regulation of hematopoietic homeostasis.

Introduction

In the previous chapter, we explored the function of *O*-fucose dependent Notch signaling pathway in lymphoid versus myeloid lineage determination using Notch1- and Pofut1-deficient murine embryonic stem cell lines. Although we have learned a great deal about *O*-fucose biology from the cell based study, many questions, especially those regarding the cellular mechanisms by which deletion of Pofut1 leads to abnormal hematological development, still remain unanswered. This is partly due to the finite application of our cell-based approach. Here, we continue our quest for uncovering the biology of *O*-fucose in Notch and hematopoiesis by addressing issues using an animal model. Before delving straight into our data, let me quickly discuss some pertinent information.

Notch signaling regulates cell fate determination in multiple biological systems. There are four known Notch homologs in mammals. Each can be activated by one or more surface expressing Notch ligands on the neighboring cells. The binding domain of Notch is decorated by multiple *O*-fucose molecules, of which the additions are catalyzed by Protein *O*-fucosyltransferase 1 (Pofut1). These *O*-fucose moieties can further be elongated *N*-acetylglucosamine, a reaction catalyzed by the Fringe glycosyltransferase. These fucose-containing glycans are critical in modulating transactivation of Notch through altering the sensitivity of Notch receptors to Delta-like versus Jagged ligands.

Fringe-mediated extension of *O*-fucose has been shown to differentially influencing Notch signaling in embryogenesis of *Drosophila* and mouse (105-108).

The significance of Notch function in both T-cell lineage differentiation and splenic marginal zone B cells has been documented extensively (85). We recently published findings indicating Notch regulates myelopoiesis by inhibiting myeloid committed progenitor differentiation at the stage of common myeloid progenitor (CMP) to granulo-monocytic progenitor (GMP) conversion (86). Combined with many reports that Notch activation expands HSC, suppresses myeloid development while promoting lymphopoiesis (73-78), these findings suggest that Notch pathway plays roles in the hematopoietic stem cell biology and the decision making between lymphoid and myeloid lineages. However, the significance of Notch signaling in hematopoietic homeostasis remains to be clarified. Conditional deletion of Notch1 or Notch1 combined with Jagged1 or Notch-dependent transcription factor, CSL, and forced expression of a dominant negative Mastermind-like 1 (DNMAML) of canonical Notch-mediated transcriptional activation, do not suggest an essential role of Notch signaling in lineage specification and myelopoiesis (44, 81, 109). Findings from these studies suggest that other Notch receptors may be involved and/or Notch may regulate blood lineage homeostasis in a CSL-independent fashion.

To elucidate the role of *O*-fucose-modified Notch activity in hematopoiesis, we have previously studied FX-deficient mice that are genetically engineered to be conditionally defective in the de novo fucose synthesis but have an intact salvage pathway that uses exogenous fucose. Adult animals carrying homozygous FX-null allele yield a complete deficiency in *O*-fucosylation and Notch activity with phenotypes that

include, but not limited to, growth retardation, infertility, reduced hematopoietic stem cell count, neutrophilia and impaired thymocyte development unless fucose is supplemented in the diet (43, 86, 110). Complementarily, Ge and colleagues showed that removal of an individual *O*-fucose glycan within the ligand-binding domain of Notch1 partially impairs the synthesis of mature T cells (111). This observation suggests that multiple *O*-fucose glycoconjugates are critical to normal Notch function. However, mice containing deletion of Notch1 EGF repeats 8-12 are embryonic lethal with phenotypes indistinguishable from those of Notch1 knock-out mutants (112). Moreover, homozygous germ-line deficiency in Pofut1 results in embryonic lethality with defects in cardiovascular and neurological development. Nevertheless, the phenotypes exhibited in the Pofut1-deficient mutant are analogous to but more severe than those of mice with a single Notch receptor knockout (58), suggesting a global Notch defect evoked by Pofut1 deficiency.

To address the role of *O*-fucose moieties on Notch in postnatal hematopoiesis, we examine mice carrying targeted inducible deficiency in the Pofut1 locus (113). Reminiscent of those observed in mice maintaining a global fucosylation deficiency, homozygosity for the Pofut1-null allele exhibits low HSC count, suppressed HSC function, myeloproliferation, aberrant MZB differentiation and thymic hypoplasia in adult mice. Interestingly, all hematological abnormalities are cell-autonomous and environmentally regulated, each of which is reversed by the forced expression of constitutively active Notch molecules. Consistent with this finding, bone marrow progenitors bearing deficient Pofut1 functions fail to differentiate into mature T lymphocytes but mature neutrophils while co-culturing with Notch ligand-expressing bone marrow stromal cells *in vitro*. Further, the fucose-dependent dosage effect of

Pofut1-haploinssuficiency indicates an essential role of Pofut1 in *O*-fucose modified Notch activation in myelopoiesis.

Methods

Mice

Animal use that was pertinent to this study was approved by Case Western Reserve University Institutional Animal Care and Use Committee. Floxed Pofut1 ($Pofut1^{f/f}$) mice were acquired from Dr. Pamela Stanley (Albert Einstein School of Medicine, Bronx, NY). Mice carrying the hemizygous allele of Mx1 promoter-driven Cre recombinase (Mx-Cre) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice maintaining homozygous floxed Pofut1 and hemizygous Mx-Cre alleles (Mx-Cre /$Pofut1^{f/f}$) were generated by breeding the progeny of $Pofut1^{f/f}$ and Mx-Cre crosses together. To obtain mice carrying targeted deficiency in the Pofut1 locus ($Pofut1^{-/-}$), three doses of 250 µg of Poly (I:C) (Sigma Aldrich, St. Louis, MO) were administered intra-peritoneally every other day into mice carrying the Mx-Cre and floxed Pofut1 alleles.

Cell lines

HEK 293T cell line was maintained in DMEM supplemented with 10% FBS while OP9 stromal cells were cultured in alpha-MEM containing 10% FBS.

Genotyping

Mouse genotype was determined by PCR of genomic DNA extracted from mouse tails. For the Pofut1 allele, PCR fragment was amplified using primers: forward primer: 5'-GGGTCACCTTCATGTACAAGTGAGTG-3'; reverse primer: 5'-ACCCACAGGCTGTGCAGTCTTTG-3' (floxed-Pofut1 allele: 960 bp; WT Pofut1

allele: 700 bp and Pofut1-null allele: 300 bp). Presence of the Mx-Cre transgene allele was detected using forward primer: 5'-GCGGTCTGGCAGTAAAAACTATC-3' and reverse primer: 5'-GTGAAACAGCATTGCTGTCACTT-3' with a size of 100 bp fragment (primer sequences were provided by the Jackson Laboratory, Bar Harbor, Maine).

Antibodies

Antibodies used for cell surface marker characterization include: B220, CD21, CD23, CD4, CD8, CD11b, Gr-1, Fc-γ, CD34, Sca-1 and c-Kit. Lineage depletion was performed using biotin-conjugated antibodies: B220, CD4, CD8, CD11b, Gr-1, Ter-119, and NK1.1.

Flow cytometric analysis

Flow cytometric analysis was performed using FACS Aria (Becton Dickinson, Franklin Lakes, NJ). For analysis of surface marker expression, all samples were stained with fluorophore-conjugated antibodies in Hanks' Balanced Salt Solution (HBSS) supplemented with 0.5% BSA at 4^{o}C for 20 minutes. Appropriate isotype controls were included. Data analysis was completed using FACSDiva software (Becton Dickinson).

Bone marrow transplantation

Bone marrow transplant was performed as described previously (86). Briefly, donor-derived bone marrow cells isolated from either wild-type (WT) or Pofut1$^{-/-}$ were delivered via tail veins of lethally irradiated (9.5 Gy) WT or Pofut1$^{-/-}$ recipients. Three months after transplantation, cells from bone marrow, peripheral blood and spleen were collected for analysis.

Bone marrow transplantation with retrovirally transduced cells

Retrovirus containing constitutive active Notch1 molecules (ICN1) was prepared as described previously (110). Briefly, Phoenix E cells were transiently transfected with either ICN1 or empty pMig-EGFP bicistronic expressing constructs using the Profection mammalian transfection system (Promega, Madison, WI). Viral supernatants were collected on three consecutive days 24 hours following the transfection reaction. Retroviral infection of total bone marrow cells and bone marrow transplantation were then performed as follows: total bone marrow cells were harvested from Pofut1$^{f/f}$ and Pofut1$^{-/-}$ mice four days following the intra-peritoneal administration of 5-FU (250 µg/kg of body weight). These cells were then cultured in the growth medium, which contained 6 ng/ml of IL3, 10 ng/ml of IL6 and 10 ng/ml of SCF for 24h. Two rounds of retroviral spinfections were performed 24 hours apart. Each round, bone marrow cells were washed and resuspended in the viral supernatant containing growth medium and 4 µg/ml of polybrene. Forty-eight hours following the second round of infection, 2×10^5 infected cells along with 2×10^5 protection cells were injected into tail veins of lethally irradiated syngeneic WT or Pofut1$^{-/-}$ recipient mice. Recipients were sacrificed at four weeks after bone marrow transplant and single-cell suspensions of peripheral blood, bone marrow and spleens were prepared and analyzed.

Soluble Notch ligand binding assay

Soluble Dll4 ligand was prepared as described previously (114). Briefly, soluble Dll4 was generated from HEK 293T cells by expressing construct where cDNA encoding the extracellular domain of Dll4 was fused to the Fc region of human IgG1 in frame. Enzyme-linked immunosorbent assay was employed to measure the concentration.

Binding was performed by incubating cells with 32 nM of soluble Dll4 in HBSS supplemented with 0.1 mM of $CaCl_2$ for 20 minutes at room temperature. Results were analyzed using FACS Aria.

***in vitro* T cell differentiation assay of hematopoietic stem cells**

Total enucleated bone marrow cells from both WT and Pofut1$^{-/-}$ mice were lineage depleted and positively sorted for Sca-1 and c-kit surface markers to obtain purified hematopoietic stem cells (LSKs). T cell differentiation assay of these LSKs was then performed as described previously (93). Approximately 500 LSKs were then cultured onto a monolayer of OP9-Ret10 (vector control) or OP9-Dll4 expressing cells in the presence of 5ng/ml of mIL-7 and 5 ng/ml of mFlt3-L for 21 days. Culture medium was replaced every third day while cells were reseeded onto a new monolayer of OP9 cells every six days. Cells were analyzed for surface expressions of mature myeloid and lymphoid markers using flow cytometry.

Quantitative RT-PCR

Freshly isolated LSKs from both WT and Pofut1$^{-/-}$ mice were first co-cultured with either OP9-control or OP9-Dll4 expressing cells for two days. Total RNA and cDNA of cultured LSKs were prepared sequentially using RNeasy Mini Kit (Qiagen, The Netherlands) and iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's specifications. Quantitative real-time PCR analyses for Notch downstream transcripts, including Deltex1, Gata-3 and Hes1were performed using Bio-Rad CFX Real-time PCR Detection System.

Statistical Analysis

Data are reported in the format of mean ± standard deviation. For statistical analysis, paired two-sided t-test was performed for continuous variables using Microsoft Excel program. The confidence level was set at $p<0.05$.

Results

Enhanced myeloproliferation and impaired lymphopoiesis in mice lacking Pofut1

Our recent studies disclose a myeloproliferative phenotype, hematopoietic stem cell (HSC) dysfunction and abnormal Notch signaling in mice maintaining a deficiency in global fucosylation. To determine the specific role of Notch *O*-fucosylation in hematopoiesis and HSC function, we sought to determine if deletion of Pofut1 would result in a similar phenotype. Since germ-line Pofut1 deletion is lethal (58), we generated Mx-Cre/Pofut1$^{f/f}$ mouse (Figure 3-1a) (115). The deletion of Pofut1 (Pofut1$^{-/-}$) was nearly completed in the marrow one month after the injection of Poly (I:C) when the Mx-Cre/Pofut1$^{f/f}$ mice developed neutrophilia in the periphery ($6.0 \pm 2.0 \times 10^3/\mu l$ versus control, $0.7 \pm 0.2 \times 10^3/\mu l$). Peripheral neutrophilia was accompanied by a decrease of both the percentage and absolute numbers of T lymphocytes. While the percentage of B lymphocytes was decreased (Figure 3-1b), its absolute number was not changed (data not shown). There was an expansion of mature granulo-monocytic cells and myeloid progenitors but a decrease of T and B lymphocytes in the marrow (Figure 3-1c). In addition, Mx-Cre/Pofut1$^{f/f}$ mice showed splenomegaly with an infiltration of mature granulo-monocytic cells and myeloid progenitors (Figure 3-1d).

A further characterization of the lymphoid compartment revealed that the thymus is drastically decreased in size when compared to the control (total thymocytes: 0.5 ± 0.3

$\times 10^6$; control: $45 \pm 4.8 \times 10^6$) (Figure 3-2a), reminiscent of the hypoplastic thymi found in FX-deficient mice while on the regular mouse chow. Flow cytometric analysis revealed that relative and absolute mature T cell subsets ($CD4^+$, $CD8^+$, and $CD4^+CD8^+$) counts were significantly reduced in Mx-Cre/Pofut1$^{f/f}$ mice while the immature DN ($CD4^-CD8^-$) thymocytes were relatively increased (Figure 3-2b). The vast majority of immature thymocytes were at the DN1 stage ($CD44^+CD25^-$). The numbers of $B220^+$ lymphocytes were mildly increased in the thymus (Figure 3-2b). In the spleen, the percentage of B and T lymphocytes were decreased while the frequency of granulo-monocytic cells was increased (Figure 3-1d). A further analysis of B lymphoid compartment in the Mx-Cre/Pofut1$^{f/f}$ mice revealed no obvious blockage of B progenitor development (data not shown); however, there was a 10-fold decrease of $IgM^{hi}IgD^{lo}$ B splenocytes comprised of type1 transitional (T1) and marginal zone B cells (MZB). There was a 34-fold decrease of $CD21^{high}CD23^{lo/-}$ MZB cells within the $B220^+$ population when compare to the controls (Figure 3-2c).

In summary, the faulty T cell development in the thymus and the suppressed MZB compartment in Mx-Cre/Pofut1$^{f/f}$ mice closely recapitulated the observed phenotypes in Notch1- and Notch2-knockout mice, respectively. A combined feature of aberrant lymphoid development and myeloproliferation, however, is reminiscent of findings observed in $FX^{-/-}$ mice, indicating that Pofut1 modulates Notch signaling in both lymphoid and myeloid lineages.

Both cell-autonomous and environmental cues contribute to the aberrant hematologic development in mice lacking Pofut1 alleles

Genetic traits are often intricately regulated by both cell-autonomous events and environmental factors. To discriminate between these two processes for our specific traits, we performed bone marrow transplantations where WT and Pofut1$^{-/-}$ donor cells were delivered into lethally irradiated WT and Pofut1$^{-/-}$ recipients via a tail vein injection. Three months post bone marrow transplant, enucleated cells were harvested for immunophenotyping from different hematologic organs, including the spleen, peripheral blood and thymus. Visually, spleens were markedly enlarged in mice transplanted with Pofut1$^{-/-}$ progenitors regardless of the recipient genotype. This increase in splenic size was positively correlated with an increase in total splenocyte count. Characterization of cellular composition of these enlarged spleens showed an enhanced proportion of the Gr-1^{+} myeloid compartment and a corresponding reduced mature T-lymphoid compartment (Figure 3-3a). In comparison, recipients of WT donor cells yielded a larger percentage of mature T lymphocytes and a smaller fraction of granulocytes without any significant increases in absolute cell number. This myeloproliferative phenotype was also observed in the peripheral blood of those who received Pofut1$^{-/-}$ donor cells. The absolute neutrophil counts per micro-liter of peripheral blood were significantly increased in mice receiving Pofut1$^{-/-}$ bone marrow cells by three- to five-fold when compared with recipients injected with WT cells (Figure 3-3a). Enhanced neutrophil count in the periphery was the most profound in recipients bearing Pofut1-null alleles.

To examine the development of the T lymphocyte compartment, single cell suspensions of thymi from individual recipients were counted and characterized. Total thymocyte counts in WT and Pofut1$^{-/-}$ mice that have received Pofut1$^{-/-}$ marrow were reduced by at least an order of magnitude when compared with those that received WT

cells (Figure 3-3b). The Marked decrease in total thymocyte count in these mice was largely due to the absence of mature T lymphocytes. While mice who received WT bone marrow cells maintained a typical thymocyte staining profile where the vast majority of cells are either single- or double-positive ($CD4^+/CD8^+$), mice receiving $Pofut1^{-/-}$ donor progenitors yielded either a limited number of double-positive (WT recipients) or double-negative immature thymocytes ($Pofut1^{-/-}$ recipients) (Figure 3-3b). In our bone marrow transplantations, hematologic traits were the most severe in $Pofut1^{-/-}$ mice that received $Pofut1^{-/-}$ bone marrow donor cells while WT recipients yielded a relatively milder neutrophilia and thymic hypoplasia. Taken together, our findings suggest that deranged Pofut1-dependent phenotypes in the hematopoietic compartment are primarily cell autonomous although environmental factors appear to play a role as well.

Although it appeared that $Pofut1^{-/-}$ progenitors were capable of interacting with wild type thymic stromal environment to evolve into mature thymocytes, their T lymphopoiesis was more severely disturbed in WT recipients who received equal numbers of $Pofut1^{-/-}$ donor cells (CD45.2) and WT competitor cells (CD45.1) in the competitive transplantation (Figure 3-4a, b). The percentages of CD45.2 $Pofut1^{-/-}$ donor cells in total thymocytes were from 0.8% to 40% (mean18.6 $\pm$ 19.4%, n=4). The absolute number of $Pofut1^{-/-}$ donor-derived single positive $CD4^+$ or $CD8^+$ cells, as well as DP cells, were much less represented when compared to WT competitor cells (Figure 3-4a, b), whereas the DN cells were relatively increased with the majority of those residing in the DN1 stage (Figure 3-4b). In the periphery, the chimerism established by $Pofut1^{-/-}$ donor cells in the presence of equal number of competitors was in the range of 49% to 60%. The majority of $Pofut1^{-/-}$ donor-derived cells were neutrophils, while T lymphocyte

compartment was suppressed and B lymphocytes were slightly increased when compare to those derived from the competitors (Figure 3-4c). The increased neutrophil numbers derived from Pofut1$^{-/-}$ donor cells were accompanied by an expansion of Gr1^{+} granulocytes and myeloid GMPs (data not shown) in the marrow, and splenic infiltration of granulocytes (Figure 3-4d). Although Pofut1$^{-/-}$ donor derived T cells were relatively decreased while B cells were relatively increased in the spleen, the Pofut1$^{-/-}$ MZB cells were decreased by ~50%.

Taken together, our findings suggest that deranged Pofut1-dependent phenotypes in the hematopoietic compartment are both cell and environment dependent. Further, marrow progenitors of Pofut1$^{-/-}$ genotype display enhanced myeloid potential in both non-competitive and competitive settings, but manifest more severe T lymphoid development defects in competitive reconstitution experiment.

Hematopoietic progenitors deficient in Pofut1 exhibit suppressed lymphopoiesis in Notch-dependent T cell differentiation *in vitro*

Because *O*-fucosylation is essential for normal function of Notch activity, in an effort to delineate the hematological consequences of induced *O*-fucosylation deficiency in Pofut1$^{-/-}$ mice, Notch functions were assessed in hematopoietic stem cells isolated from these mice. We first employed an *in vitro* Notch-dependent T cell induction assay where freshly purified LSKs were co-cultured with bone marrow stromal cells bearing Notch ligand Dll4 in the presence of IL-7 and Flt3-L, a T-lymphoid polarizing condition. Twenty days following the initiation of co-culture, WT LSKs gave rise to CD8^{+} T cells with the support of OP9-Dll4 while mature T lymphocytes were virtually undetectable

from LSKs carrying Pofut1$^{-/-}$ alleles (Figure 3-5a). Instead, the vast majority of these hematopoietic progenitors differentiated into a Gr-1^{+} myeloid lineage (62%). To confirm that productive Notch activity was essential to the establishment of mature T cells, LSK-OP9 co-cultures were supplemented with 10 μM of gamma secretase inhibitor, a canonical Notch signaling blocker. In the absence of Notch activity, WT LSKs progressed into Gr-1^{+} cells at the expense of mature T-lymphocytes even with the support of Dll4. Considered together, these observations suggest that suppression of T lymphopoiesis in hematopoietic stem cells bearing a defect in synthesizing *O*-fucose moieties is due to the disruption of Notch signaling.

Notch regulates cellular behaviors via controlling the differential expression of downstream gene targets. To elucidate the molecular changes of *O*-fucosylation deficiency in this context, we examined Notch ligand-mediated expressions of Notch-specific transcripts in Pofut1$^{-/-}$ hematopoietic stem cells. Notch-regulated genes encoding Deltex1 and Hes1 were quantified in freshly isolated LSKs from either WT or Pofut1$^{-/-}$ mice that were briefly cultured with either OP9-control or OP9-Dll4. Expression levels of all three transcripts examined were down-regulated in Pofut1$^{-/-}$ bone marrow progenitors by two- to four-fold when compared with WT cells (Figure 3-5b). They were, however, not completely diminished.

We next sought to explore cellular mechanism, by which Notch activity is reduced in bone marrow progenitors bearing deficient Pofut1 functions. *O*-fucose moieties have been implicated in normal Notch receptor-ligand interaction. To address this possibility, purified LSKs from both WT and Pofut1$^{-/-}$ mice were subjected to soluble Dll4 binding, of which binding strength is expressed as mean fluorescence intensity of

ligand staining. When compared with human IgG staining, staining of WT LSKs with soluble Dll4 was increased by approximately 12-fold (MFI ratio: 3894/302) whereas staining of Pofut1$^{-/-}$ LSKs was 1.9-fold (MFI ratio: 479/256) over the control (Figure 3-5c). Reduced soluble Dll4 staining in the absence of Pofut1 function did not, however, account for the possibility that Notch receptors without *O*-fucosylation are not trafficked properly to the cell surface. Immunophenotyping of Notch expression using antibodies specific to an individual homolog revealed that both Notch1 and Notch2 were modestly decreased in the absence of Pofut1 function while Notch3 maintained its expression level (Figure 3-5d). Notch4 was not expressed in LSKs. Regardless of the specific mechanisms, our findings indicate that soluble Dll4 inadequately interacts with Notch receptors lacking *O*-fucose moieties and thus leads to reduced expression of Notch-specific transcripts. Altogether, our results suggest that myeloproliferation and mature T lymphocyte hypoplasia in Pofut1$^{-/-}$ mice are results of anomalous Notch signaling.

Rescuing faulty hematopoietic development in Pofut1-null mice

In an effort to reverse myeloproliferation and faulty T lymphocyte development in Pofut1$^{-/-}$ mice, we reconstituted Pofut1$^{-/-}$ hematopoietic progenitors with either active Notch1 molecules (ICN1) or vector control (pMig) and transplanted them into lethally irradiated recipients, along with protective cells (Figure 3-6a). Four weeks following transplantation, donor-derived blood cells were isolated from the bone marrow, the peripheral blood and the spleen for immunophenotyping with respect to expression of lineage-markers. Figure 3-6b summarizes the lineage distribution of donor-derived cells in the periphery of each recipient as a function of plasmid transduced into donor

progenitors. Although the proportion of cells bearing a lineage marker of interest varied for each recipient, in general, donor cells transduced with the pMig vector largely differentiated into Gr-1$^+$ myeloid cells regardless of recipient genotype. In comparison, donor cells that were forced to constitutively express active Notch1 gave rise to CD8$^+$ lymphocytes instead of neutrophils. Together, these data indicate that constitutively active Notch1 molecules efficiently reverse the aberrant hematopoietic development of progenitors carrying homozygous null alleles in the Pofut1 locus. Moreover, these findings imply that *O*-fucose-dependent Notch activity plays a functional role in lineage specification between lymphopoiesis and myelopoiesis during development.

Discussion

In this study, we demonstrated that insufficient function of protein *O*-fucosyltransferase 1 leads to aberrant B-, T- and myeloid-lineage differentiation in adult mice. These phenotypic traits of hematologic relevance are reminiscent of those observed in mice engineered to be completely deficient in cellular fucosylation. Reversal of these phenotypes by reconstituting bone marrow progenitors from both mouse strains with constitutively active Notch1 molecules strongly suggest a role for *O*-fucose dependent Notch activity in the formation of mature blood cells. Our finding reiterates the significance of Notch in lymphoid lineage specification although discrepancies in myelopoiesis among studies still remain to be resolved.

The roles of Notch1 and Notch2 in T-cell lineage commitment and marginal zone B cell formation are well established, respectively (44, 104). Thus, it is not surprising that the development of both lymphoid compartments, defined phenotypically, is severely

compromised in mice with Pofut1 deficiency. In the thymus, the vast majority of cells are double-negative immature thymocytes bearing CD44 surface marker, suggesting that the temporal requirement of Notch activity for T lineage commitment occurs probably soon after the thymic homing of progenitors or any time prior to the entry. Although we cannot rule out the possibility of pre-thymic T-lineage commitment, considered together with the non-redundant role of thymic Dll4 ligand in T cell development (69, 116), our findings imply that the entry into the thymus is critical for the thymic progenitors to receive proper Notch signaling and thus obligate to T cell specification.

The process of thymic homing is intricately modulated by multiple molecular events. Several trans-membrane proteins and their associated molecules pairs have been identified to facilitate this process, including CCR9, CD44 and PSGL-1. Both Notch1 and lunatic fringe (Lfng) glycosyltransferase have also been implicated in the access of thymic progenitors to intra-thymic niche (117, 118). Thus, it is possible that *O*-fucose moieties provide the necessary mechanism for thymic progenitors anchoring to the thymic niche. Our data demonstrate that Pofut1-deficient donor progenitors are unable to differentiate into mature T cells in the wild-type recipients only if they are competing against wild-type donor cells. Given that Lfng is presence in the thymic progenitors (118) and its modification enhances the interaction between Notch and ligands, these findings suggest that *O*-fucosylation of Notch facilitates the entry of thymic progenitors into the thymus by providing these cells with a competitive advantage binding to the thymic stroma. This intriguing possibility for the new role of Notch in the contribution to T lineage commitment requires further study to determine its physiological relevance.

Previous studies indicate that Notch1 signaling cell-autonomously modulates T cell differentiation (44). Although it is tempting to speculate that *O*-fucose moieties modulate T lymphopoiesis in a similar fashion, providing these glycans modify both Notch receptors and their interacting ligands in the thymic microenvironment (119), we propose that the contributions of *O*-fucosylation to T specification are both cell-autonomous and environmental. Indeed, our bone marrow transplant data show that targeted deficiency of Pofut1 in thymic progenitors leads to faulty T cell development only if they are presented with an environment lacking Pofut1. This observation, together with the requirement of *O*-fucose for Notch-ligand interaction (120) and the non-functional role of Notch receptors in the environment to thymopoiesis, suggests that proper Notch signal strength driving thymic progenitors to T lineage is equally contributed by Notch and ligands expressing in the thymic stroma, namely Dll4 and possibly Jagged2 (69, 116). Although there has been no report to date suggesting a functional property of *O*-fucose glycans in Notch ligand, disruption of *O*-fucose containing motif of Notch ligand perturbs the normal Notch function (121). Thus, further study will be required to address the role of *O*-fucose moieties on Notch ligands in Notch signaling.

Signaling through Notch receptors is critical in the formation of mature T cells although its role in myelopoiesis is slowly emerging. Coupling with our previous study where adult mice harboring global defect in fucose-dependent Notch activation leads to severe myelopoiesis, our study exemplifies that Notch signaling indeed inhibits the formation of myeloid cells. Our findings are supported by several previous studies examining whether and how different aspects of Notch signaling regulates myeloid

specification, each of which showed that constitutive Notch1 activity prevented myeloid progenitors from further differentiation into mature granulocytes (73-77) and inactivation of all Notch ligands in the marrow microenvironment led to myeloproliferative disease (78). In contrast, earlier works showed that active Notch1 and Notch2 enhanced granulopoiesis in a myeloid progenitor cell line (32D Cl3) (79, 80). This difference may be due to the level of commitment of a progenitor line to the terminal lineage in which the role of Notch activity is studied. The growth of 32D Cl3 is maintained by exogenous IL-3. Withdrawal of IL-3 and supplement of granulocyte-colony stimulating factor supports the terminal differentiation of these progenitors into mature granulocytes (122). Thus, constitutive Notch activity possibly provides survival and proliferation signals to 32D Cl3, which accelerates their pre-determined path to the mature granulocytic lineage. In other studies, the deletion of individual component in the Notch ligand-receptor axis, namely Notch1 and Notch2, showed no obvious defect in the myeloid compartment. This discrepancy may simply stem from the redundant function of Notch homologs in the marrow progenitors. To confirm our finding of Notch regulation in myelopoiesis, functional correlates of compound Notch homologs deficiency to the modulation of lymphoid versus myeloid lineage diversification are needed.

Despite some suggestions to the contrary, we provide evidence supporting the functional role of *O*-fucose dependent Notch activity in controlling the specification of blood lineages in adult mice. Our data further illustrating that Notch signaling regulates the lineage differentiation by upregulating specific transcriptional targets while downregulating others. The expression of these genes controls cell behavior, which gives the lineage its characteristics. Many of these transcripts are expressed in a CSL-

dependent manner, including Deltex, Gata3, Hes family genes and pre-Tα. Although their expressions are enhanced by Notch activation, how and whether each transcript promotes T lymphopoiesis and suppresses myelopoiesis remains unclear. These cellular mechanisms are critical to our understanding towards the functional roles of Notch activity in T lymphopoiesis. It is evident that studies aimed to address these issues are warranted.

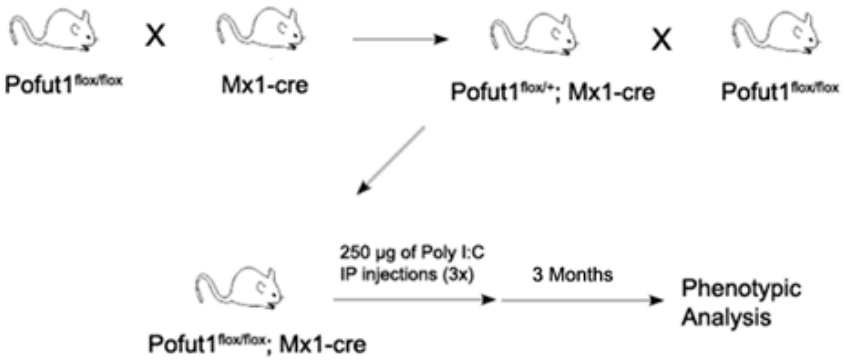
Notes to Chapter III

I want to express my gratitude to Yuanshuai Huang, Jay Myers and Xiaoran Huang for their wonderful technical assistance. I want to thank Dr. Wei Xin for reviewing the microscopic anatomy of the spleen specimens. I also want to thank Dr. Lan Zhou for her critical analysis of data and constructive suggestions during the preparation of the manuscript for this study. Retroviral vector containing the constitutive active Notch1 domain was previously cloned by Bronislawa Petryniak.

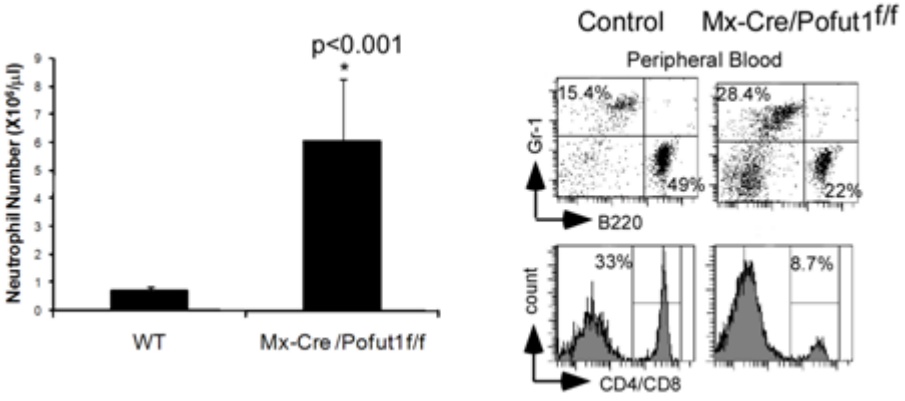
Figure 3-1. Enhanced neutrophil compartment in Mx-Cre/Pofut1$^{f/f}$ mice. a. Schematic diagram depicting the generation of mice maintaining induced targeted deficiency in the Pofut1 locus. **b.** Peripheral neutrophil counts for WT and Mx-Cre/Pofut1$^{f/f}$ mice (n=6). Cellular distribution of B (B220^{+}), T (CD4/8^{+}) and neutrophil (Gr-1^{+}) lineages in the periphery. **c.** Percentage of B-cells, T-cells and neutrophils in the bone marrow. **d.** FACS analysis of myeloid progenitors (CMP: Lin^{-}c-kit^{+}Sca-1^{-}IL7R^{-}CD34^{+}FcγRII^{low}; GMP: Lin^{-}c-kit^{+}Sca-1^{-}IL7R^{-}CD34^{+}FcγRII^{+}; MEP: Lin^{-}c-kit^{+}Sca-1^{-}IL7R^{-} CD34^{low}FcγRII^{low}). Populations of CMP or GMP are shown as percentages of Lin^{-}Sca-1^{-}IL7R^{-}c-kit^{+} cells. **e**. Gross and microscopic anatomy of the spleen. Hematoxylin and Eosin staining of spleen specimens from both WT and Pofut1-deficient mice. FACS analysis of B-cells, T-cells and neutrophils in the spleen. All Mx-Cre/Pofut1f/f mice received Poly (I:C) injections three 250 µg/dose every other day.

Figure 3-1

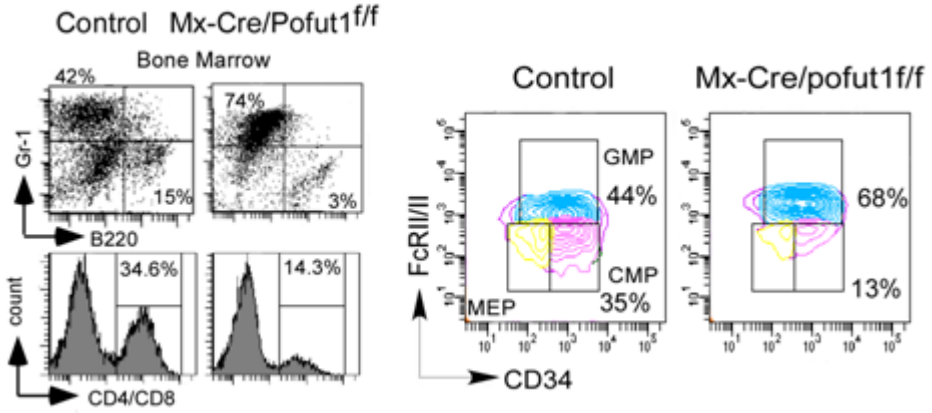
a.


Pofut1flox/flox
X
Mx1-cre
Pofut1flox/+; Mx1-cre
X
Pofut1flox/flox
250 µg of Poly I:C
IP injections (3x)
3 Months
Phenotypic
Analysis
Pofut1flox/flox; Mx1-cre

b.


p<0.001
*
Neutrophil Number (X10⁶/µl)
WT
Mx-Cre /Pofut1f/f
Control
Mx-Cre/Pofut1f/f
Peripheral Blood
15.4%
49%
28.4%
22%
Gr-1
B220
33%
8.7%
count
CD4/CD8

c.


Control
Mx-Cre/Pofut1f/f
Bone Marrow
42%
74%
Gr-1
15%
3%
B220
34.6%
14.3%
count
CD4/CD8
Control
Mx-Cre/pofut1f/f
GMP
44%
68%
FcRII/III
CMP
35%
MEP
13%
CD34

d.

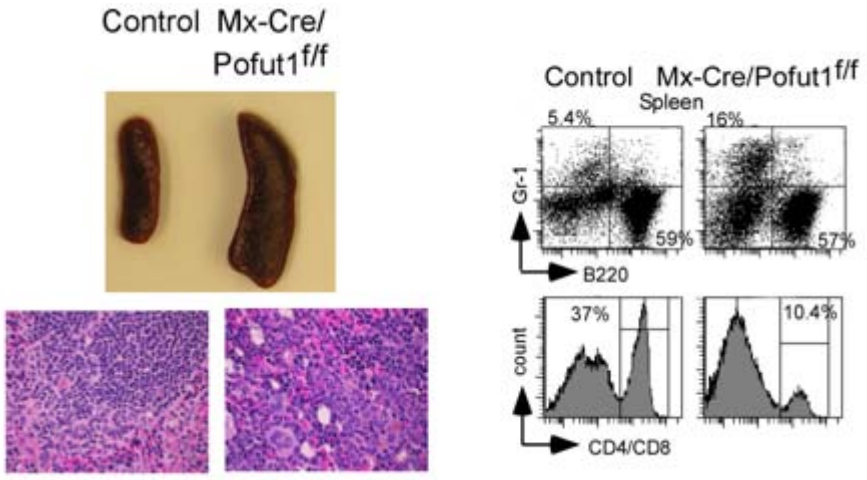
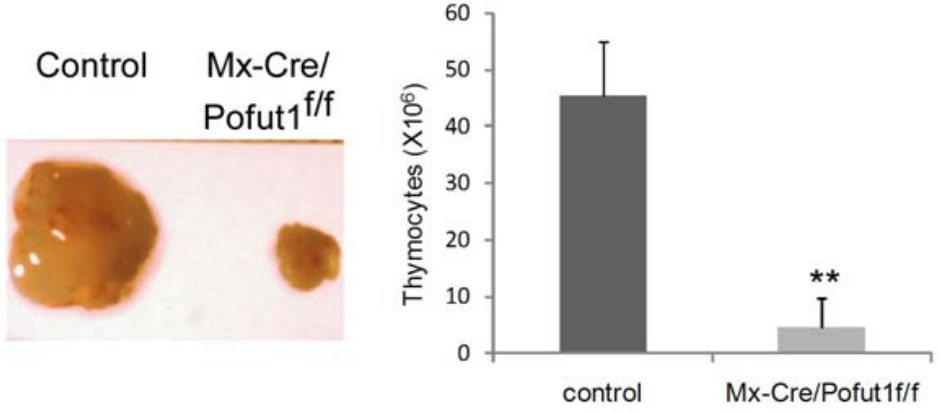
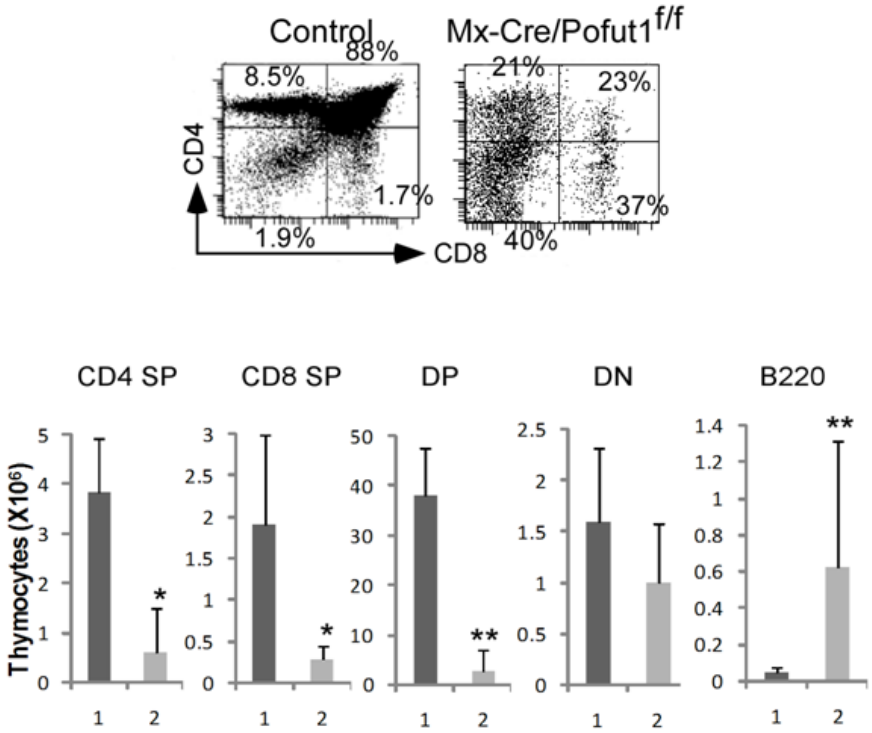

Control
Mx-Cre/
Pofut1f/f
Control
Mx-Cre/Pofut1f/f
Spleen
5.4%
16%
Gr-1
59%
57%
B220
37%
10.4%
count
CD4/CD8

Figure 3-2

a.


Control
Mx-Cre/
Pofut1f/f
Thymocytes (X10⁶)
60
50
40
30
20
10
0
**
control
Mx-Cre/Pofut1f/f

b.


Control
Mx-Cre/Pofut1f/f
8.5%
88%
1.7%
1.9%
21%
23%
37%
40%
CD4
CD8
CD4 SP
CD8 SP
DP
DN
B220
Thymocytes (X10⁶)
*
*
**
**

c.

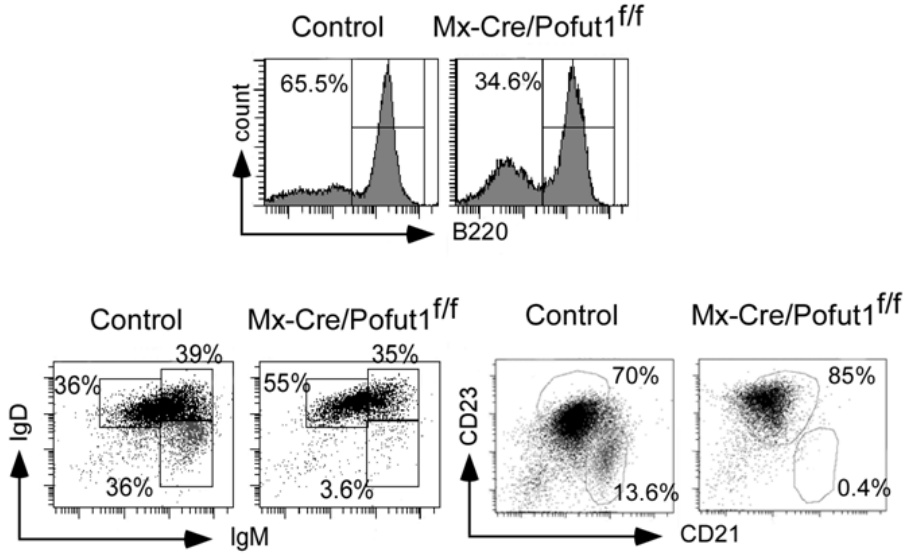
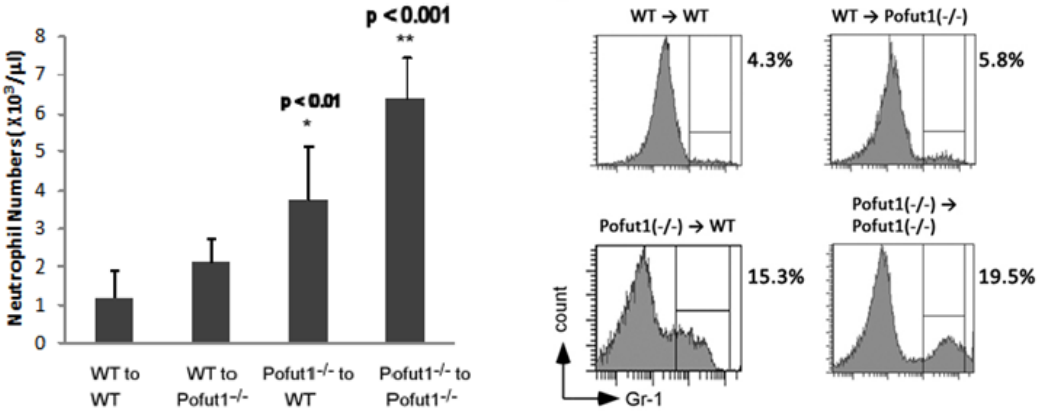


Figure 3-2. Impaired mature T cell and Marginal Zone B- and T1 B-compartments development in Mx-Cre/Pofut1$^{f/f}$ mice. a. Gross anatomy of the thymus and the total thymocyte counts from both WT and Mx-Cre/Pofut1$^{f/f}$ mice (n=6). **b.** Flow cytometric analysis of CD4 and CD8 surface markers and counts of CD4 SP (single positive), CD8 SP, DP (double positive, CD4$^+$/CD8$^+$), DN (double negative; CD4$^-$/CD8$^-$) and B220$^+$ cells. 1 – control; 2 – Mx-Cre/Pofut1$^{f/f}$ **c.** Percentage of splenic B220$^+$ cells. Flow cytometric analysis of IgD$^+$/IgM$^+$ and CD21$^+$/CD23$^+$ within the B220$^+$ population. Student *t*-test was performed to evaluate the counts of thymocytes. * $p<0.05$ and ** $p<0.01$.

Figure 3-3

a.



b.

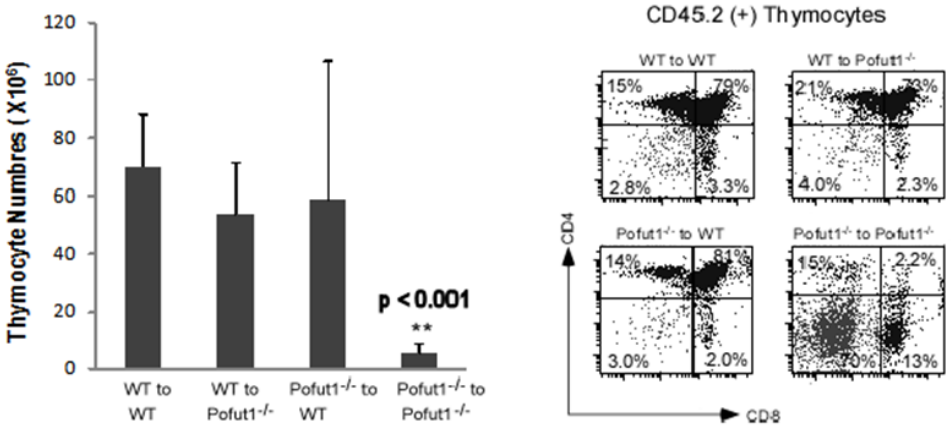
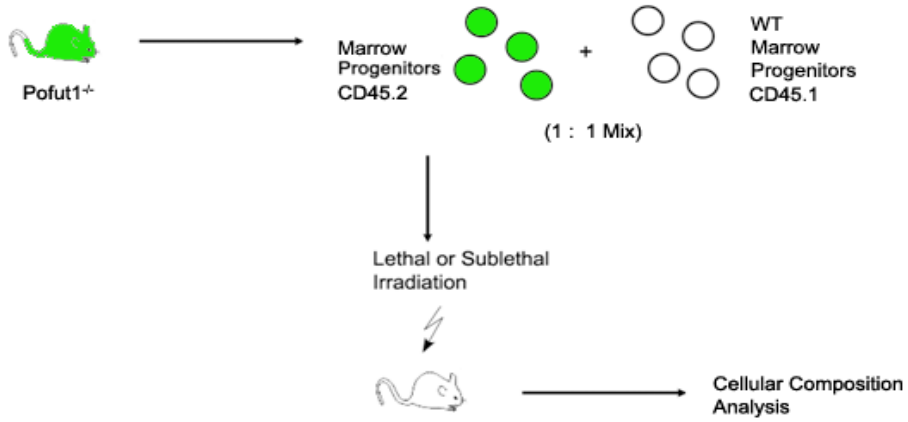


Figure 3-3. Aberrant hematological traits in Mx-Cre/Pofut1$^{f/f}$ mice (Pofut1$^{-/-}$) are both cell-autonomously and environmentally regulated. a. Peripheral neutrophil counts in WT or Pofut1$^{-/-}$ mice three months after receiving WT or Pofut1$^{-/-}$ progenitors. Histograms of the peripheral neutrophil distribution. **b.** Total thymocyte counts three months following the bone marrow transplant and scatter plots of CD4 and CD8 surface marker analysis.

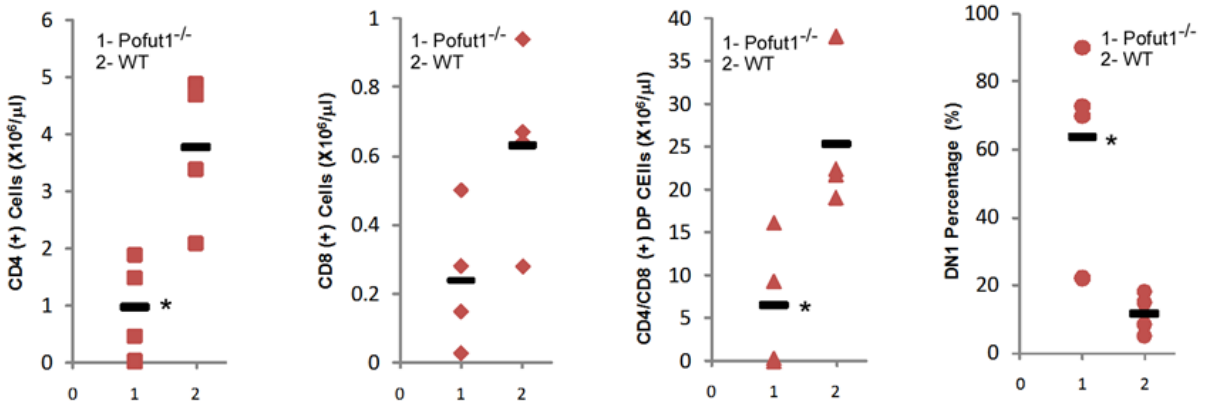
Figure 3-4. In competing against wild-type cells, Pofut1$^{-/-}$ progenitors lead to more severe hematological phenotypes. a. Cartoon showing our experimental setup for the competitive transplantation experiment. **b.** Absolute count of CD4+, CD8+, DP (double positive) thymocytes and percentage of DN1 thymocytes derived from either Pofut1$^{-/-}$ or the competitive WT progenitors three months following the transplant. 1 – Pofut1$^{-/-}$ progenitors; 2 – WT competitors (n=4) **c.** Peripheral neutrophil and mature T cell counts. **d.** Percentage of splenic granulocytes, T cells, B cells and marginal zone B cells derived from either Pofut1$^{-/-}$ or WT progenitors. Statistical analysis was done using student *t*-test. * $p<0.05$.

Figure 3-4

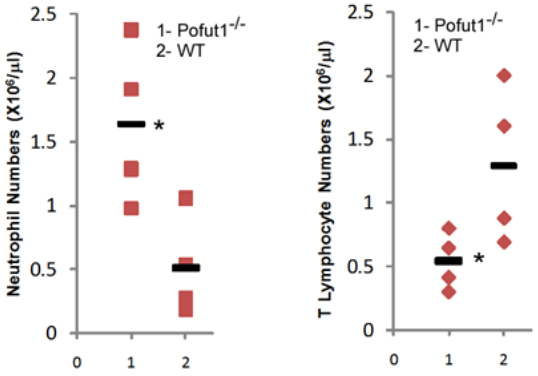
a.


Pofut1-/-
Marrow
Progenitors
CD45.2
+
WT
Marrow
Progenitors
CD45.1
(1 : 1 Mix)
Lethal or Sublethal
Irradiation
Cellular Composition
Analysis

b.


1- Pofut1-/-
2- WT
CD4 (+) Cells (X10⁶/µl)
CD8 (+) Cells (X10⁶/µl)
CD4/CD8 (+) DP CEIls (X10⁶/µl)
DN1 Percentage (%)
*

c.


Neutrophil Numbers (X10⁶/µl)
2.5
2
1.5
1
0.5
0
0
1
2
1- Pofut1-/-
2- WT
*
T Lymphocyte Numbers (X10⁶/µl)
2.5
2
1.5
1
0.5
0
0
1
2
1- Pofut1-/-
2- WT
*

d.

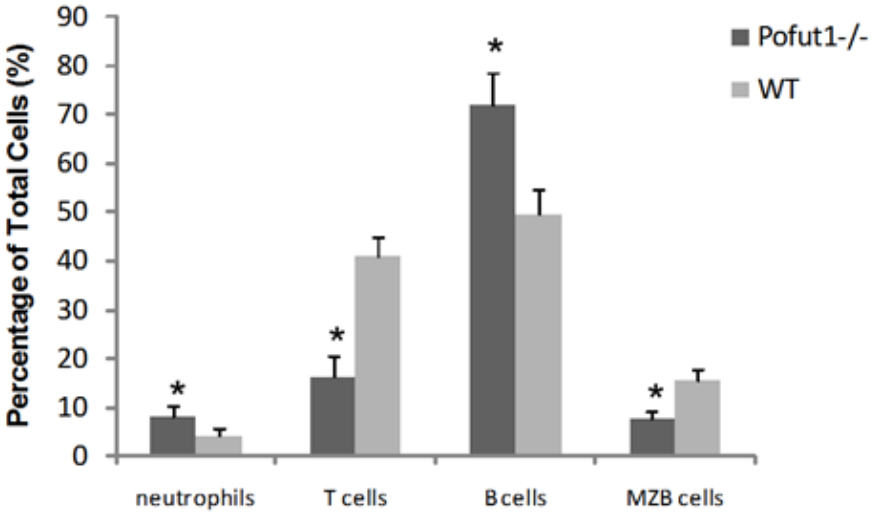
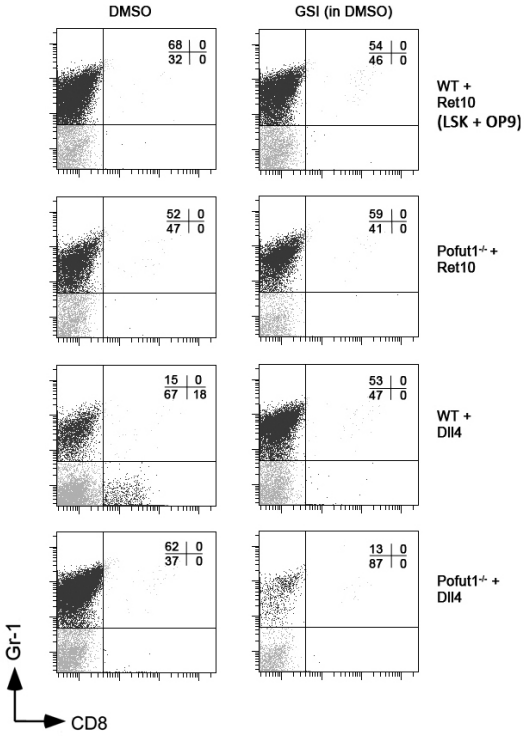

Percentage of Total Cells (%)
90
80
70
60
50
40
30
20
10
0
*
*
*
*
neutrophils
T cells
B cells
MZB cells
Pofut1-/-
WT

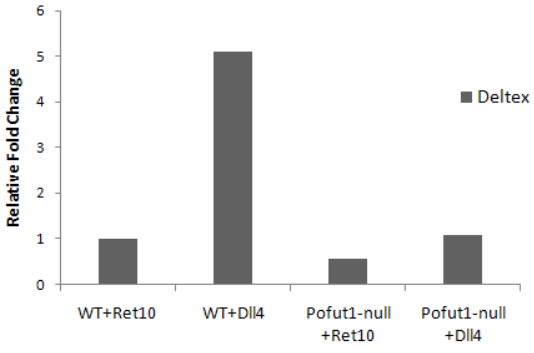
Figure 3-5. Pofut1$^{-/-}$ progenitors have suppressed lymphopoiesis but enhanced myelopoiesis *in vitro.* This may be due to reduced interaction with ligand and the surface expression of Notch receptors. a. Flow cytometric analysis of Gr-1 and CD8 surface markers for Lin^{-}c-kit^{+}Sca-1^{+} (LSK) progenitors following 20 days of co-culturing with OP-9 bone marrow stromal cells bearing either Dll4 or no ligand (Ret10) in the presence of either gamma secretase inhibitor (GSI, dissolved in DMSO) or DMSO (control) . **b.** Representative quantitative RT-PCR of Deltex1 or Hes1 from LSK progenitors following a four-day OP-9 co-culturing. **c.** Staining of LSK cells derived from WT or Pofut1$^{-.-}$ with soluble Dll4 or human IgG (control). **d.** Representative flow cytometric analysis of the Notch1 and Notch2 expressions on the surface of WT and Pofut1$^{-/-}$ LSK cells.

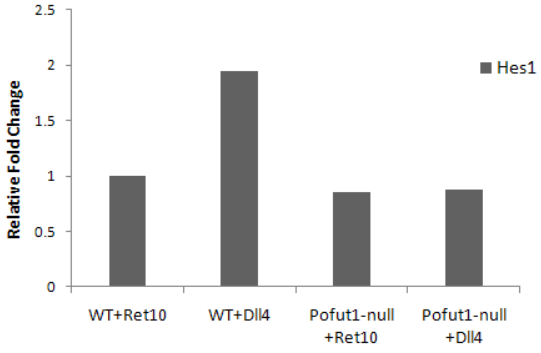
Figure 3-5

a.

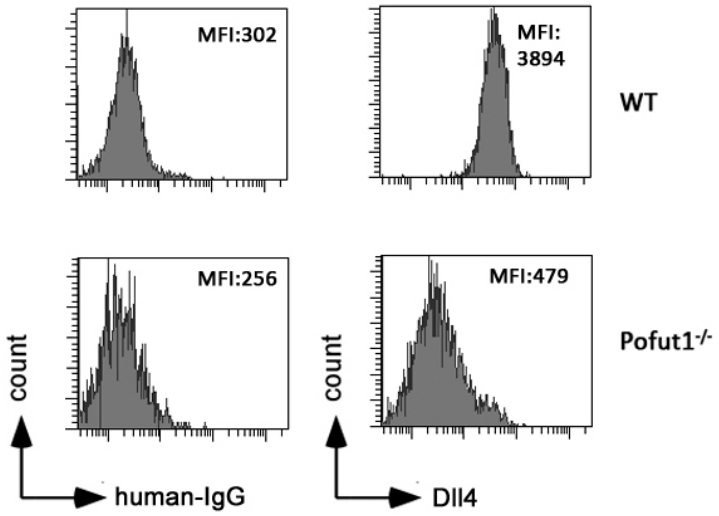

DMSO
GSI (in DMSO)
68 | 0
32 | 0
54 | 0
46 | 0
WT + Ret10 (LSK + OP9)
52 | 0
47 | 0
59 | 0
41 | 0
Pofut1-/- + Ret10
15 | 0
67 | 18
53 | 0
47 | 0
WT + Dll4
62 | 0
37 | 0
13 | 0
87 | 0
Pofut1-/- + Dll4
Gr-1
CD8

b.


Relative Fold Change
Deltex
WT+Ret10
WT+Dll4
Pofut1-null +Ret10
Pofut1-null +Dll4


Relative Fold Change
Hes1
WT+Ret10
WT+Dll4
Pofut1-null +Ret10
Pofut1-null +Dll4

c.


MFI:302
MFI: 3894
WT
MFI:256
MFI:479
Pofut1-/-
count
human-IgG
count
Dll4

d.

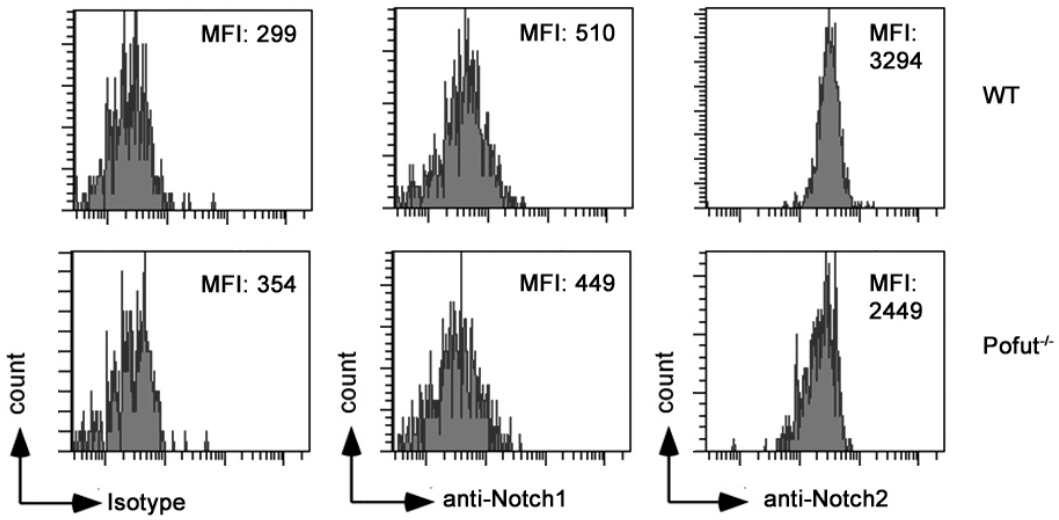
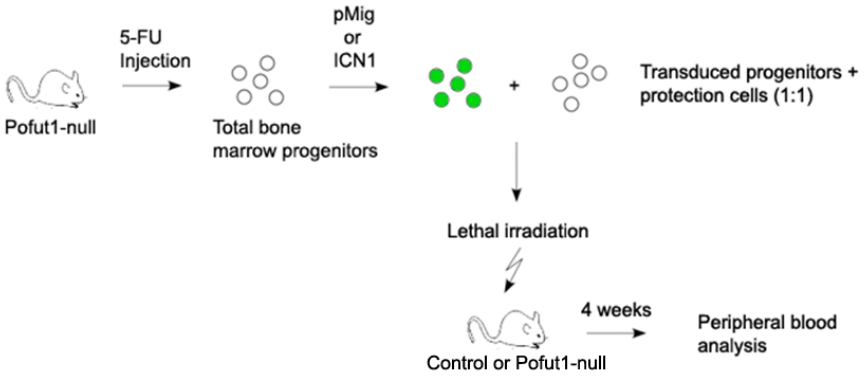

MFI: 299
MFI: 510
MFI: 3294
WT
MFI: 354
MFI: 449
MFI: 2449
Pofut-/-
count
Isotype
count
anti-Notch1
count
anti-Notch2

Figure 3-6

a.



b.

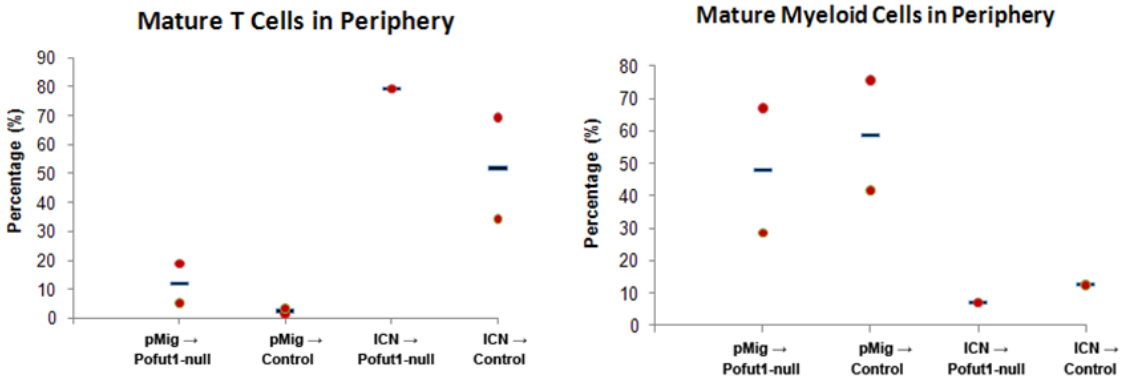


Figure 3-6. Aberrant hematologic phenotypes in Pofut1-/- mice were reversed with bone marrow progenitors expressed constitutively active Notch1. a. Schematic diagram illustrating our experimental approach for reconstituting Pofut1$^{-/-}$ marrow progenitors with active Notch1 molecules or vector control (pMig) prior to injecting into recipients. **b.** Percentage of positively transduced cells expressing mature T cells (CD4/CD8) or mature myeloid cells (Gr-1) surface markers in the periphery four weeks following the transplantation.

CHAPTER IV

Regulation of Individual *O*-Fucose in Ligand-mediated Notch1 Signaling

Abstract

We have so far demonstrated that *O*-fucosylation of Notch plays an essential role in Notch activity and the normal development of mature blood cells. As described in Chapter I, the extracellular domain of Notch is composed of multiple tandem EGF-like repeat motifs, each of which contains serine/threonine residues that may be decorated with *O*-fucose molecules. These *O*-fucose moieties can be further elongated by Fringe glycosyltransferases, which append *N*-acetylglucosamine molecules to fucose through a covalent β-1,3-linkage. Although we have assigned *O*-fucose glycoconjugates on Notch a physiological role in hematopoiesis, it remains unclear whether all or a subset of these molecules are critical to Notch function. To address this issue, we first showed that Fringe elongation of *O*-fucose on Notch1 enhances the binding affinity between Notch1 and Delta-like ligands including Delta-like-1 (Dll1) and Delta-like-4 (Dll4). Furthermore, this modification up-regulates Dll1- and Dll4-mediated Notch signaling. On the contrary, the Fringe modification does not appear to regulate the Notch1-Jagged (J1 or J2) interaction and thus Jagged-mediated Notch signaling. We then examined the induction of Notch signaling in the presence of Lunatic Fringe after an individual *O*-fucose site was abolished. Our results suggest that multiple *O*-fucose sites on Notch1 differentially participate in Dll1- and Dll4-mediated Notch activation. Mutations at both EGF9 and EGF12 reduced Notch activity significantly. We further examined the regulation of

individual *O*-fucose sites in Notch-ligand binding. When comparing with the wild-type control, the affinity of soluble Dll1, Dll4, and Jagged-2 ligands to Notch1 expressing cells was reduced in the presence of individual EGF9 and EGF12 mutations. Moreover, ablations of multiple *O*-fucose sites dramatically reduced the surface expression of Notch1. Together, these findings indicate that a subset of *O*-fucose sites play an essential role in the activation of Notch signaling.

Introduction

Notch signaling is regulated by a series of post-translational modifications of Notch (123), including the modification of Notch receptors by *O*-fucosylation. We and others have demonstrated that these *O*-fucose moieties play a critical role in regulating the normal activity of Notch and thus Notch-dependent developmental biology. The extracellular domain of Notch is composed of many tandem epidermal growth factor-like (EGF) repeat domains. There are 36 of them on Notch1. Each EGF domain contains six highly conserved cysteine residues and serine/threonine residues that may be modified by *O*-fucose via a covalent bond. Previous studies have identified a consensus peptide sequence within an EGF-repeat module where the *O*-fucose site resides: $C_2X_{4-5}(S/T)C_3$ (54). The additions of fucose molecules to each EGF-repeat is catalyzed by Protein *O*-fucosyltransferase 1 (Pofut1) in the endoplasmic reticulum (87, 88). These *O*-fucose molecules can further be elongated by the addition of *N*-acetylglucosamine, a reaction catalyzed by one of the three fucose-specific β1,3-N-acetylglucosaminyltransferases (Lunatic, Manic and Radical Fringe) in the Golgi apparatus in a lineage specific manner

(59, 124). The modification of Notch by Fringe proteins potentiates Notch and Delta interactions while inhibiting Notch and Jagged binding in *Drosophila* (120)

Since several EGF domains of Notch1 are modified by *O*-fucose, it is unclear whether all *O*-fucose sites contribute to Notch activity equally. Molecular and biochemical studies have revealed three highly conserved sites (EGF12, EGF26 and EGF27) that are critical for the regulation of Delta-like-1 (Dll1) and Jagged1 (J1)-mediated Notch1 activity in COS-7 cells (125). In *Drosophila* Notch receptors carrying the ablated EGF12 *O*-fucose site yielded partially reduced activity (126) while other less conserved *O*-fucose sites, including EGF9 and EGF24, were also involved in Notch-ligand interactions in S2 cells (127). Currently the roles of individual *O*-fucosylation sites in mammalian systems in receptor-ligand binding, receptor trafficking and ligand-facilitated Notch signaling have yet to be determined. To address these issues, we have used cell-based assays to assess the efficiency of binding of the Notch ligands Delta-like-1, Delta-like-4, Jagged1 and Jagged2 to mouse Notch1 expressed with or without Fringe modification and as a function of site-directed elimination of *O*-fucosylation sites within EGF domains thought to contribute to Notch biology (domains 8, 9, 12, 24, 26 and 27). We also assessed how the individual *O*-fucosylation sites modulate the expression levels of surface Notch1. Both Binding and surface expression data from these studies were then correlated with Notch ligand-dependent signal transduction in a similar cell-based system. These studies allow us to conclude that *O*-fucosylation sites in EGF9 and EGF12 play a primary role in regulating Dll1-, Dll4- and J2-mediated Notch binding and signaling in mammalian cells. From these studies, we also conclude that elongation of *O*-fucose by the glycosyltransferase Lunatic Fringe enhances Notch-Dll binding

interactions, and Dll-dependent signaling, while inhibiting Notch-Jagged interactions and Jagged-dependent signaling. Furthermore, we conclude that the three members of the Fringe family do not significantly differ in their ability to modulate ligand binding and signaling. Finally, we conclude from these studies that *O*-fucosylation of all examined EGF domains are required for mNotch1 to achieve its normal cell surface expression level in the presence of over-expressed lunatic fringe. Our studies underscore the multiple roles for individual *O*-fucose sites, and their Fringe-dependent modification in regulating binding between mNotch1 and its ligands, and subsequent signal transduction events.

Methods

Cell lines

A full-length mouse Notch1 nucleotide whose N-terminus is tagged with a short oligonucleotide encoding the 3xFlag epitope was first cloned into pCDNA3.1-Hygro(+) vector. This construct was used as the template to generate individual *O*-fucose mutations. Using a site-directed mutagenesis kit (Stratagene, La Jolla, CA), informative serine/threonine residues within *O*-fucose sites were changed to alanines. DNA sequence analysis confirmed the presence of each missense mutation. HEK 293T cells were then stably transfected with either a wild-type or mutant 3xFlag-Notch1 construct carrying a hygromycin selection marker. Hygromycin-selected (185 ng/ml) cell lines expressing a uniform level of cell surface 3xFlag-Notch1 were obtained by flow sorting three times based on the staining level of monoclonal anti-Flag antibody (Sigma Aldrich). *Fringe and 3xFlag-Notch1 co-expressing cell lines* – wild-type Notch1 cell line was co-transfected with one of the Fringe-EYFP bicistronic expression vectors (Lunatic, Manic

and Radical Fringe) and pPUR vector at a ratio of 10:1 (*O*-fucose mutant cell lines were transfected only with Lunatic Fringe-EYFP bicistronic expressing vector). The expression of EYFP functions as the surrogate marker for the expression level of Fringe protein.

Cell lines stably expressing approximately equal amount of Notch1 (monoclonal anti-Flag antibody staining) and Fringe glycosyltransferase (EYFP expression) were generated by flow sorting three times and selected with 1 µg/ml of puromycin between each sort. Transfection reactions were performed using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Generating Soluble Notch Ligands

Soluble ligands were generated as previously described (Chapter II, see Methods). Briefly, the nucleotide encoding the extracellular domains of mouse Dll1, Dll4 and J1 was fused in frame with the Fc region of human IgG1 in pCDNA1 vector. Soluble ligands were collected from supernatants of HEK 293T cells that were transiently transfected with the ligand constructs. The concentration of each soluble Notch ligand was approximated using enzyme-linked immunoabsorbent assays (ELISA). Expression of these soluble ligands was further visualized by Western blot using horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc). J2 soluble ligand was collected from the supernatant of a stable J2 cell line culture.

Plate-bound ligand-mediated Notch signaling assays

Soluble Notch ligand, including Dll1, Dll4, J1 and J2, were immobilized to plates as described previously (128). Briefly, a non-tissue culture treated 24-well plate was

coated with 2 µg/ml of polyclonal anti-human IgG Fc antibody in PBS at 4^{o}C overnight followed by washing and blocking with 500 µl of DMEM containing 10% of FBS at 37^{o}C for 2 hours. Each well was then washed three times and incubated with either 32 nM of soluble Notch ligands or human IgG1 at 37^{o}C for another 2 hours. 1.2 X 10^5 per well of HEK 293T cells were then plated and transiently transfected with 400 ng of wild-type or *O*-fucose mutant 3xFlag-Notch1, 200 ng of Lunatic Fringe, 40 ng of Ga981-6, TP-1 luciferase reporter (a kind gift of Dr. Georg Bornkamm, Munich, Germany), and 4 ng of Renilla luciferase (pGL4.74-TK-Renilla, Promega) using lipofectamine 2000. Six hours following the transfection reaction, medium was replaced and cells were allowed to grow for another 24 hours. At the end of the culture, both *firefly* luciferase and *Renilla* luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) and Victor light luminescence plate reader (PerkinElmer). Normalized luciferase activity for each well was reported as a ratio of *firefly* luciferase activity over *Renilla* luciferase activity. Each transfection was performed in triplicate for at least two separate times.

Notch extracellular domain (ECN) dissociation assay

The extracellular domain of Notch1 was dissociated as previously described (129). Briefly, 10^6 HEK 293T cells were transiently transfected with pCDNA3.1 vector, wt-Notch1 or 3xFlag-Notch1 in conjunction with either a pZeo vector or Lunatic Fringe construct using Lipofectamine 2000. 48 hours following transfection, cells were harvested, washed twice in TBS (20 mM Tris-HCl with 150 mM NaCl and 5 mM $CaCl_2$) and resuspended in TBS containing 5 mM $CaCl_2$ and 0.5 mM EDTA. The cell suspension was incubated at room temperature for 30 minutes. The supernatant was collected

following two centrifugations at 2000 and 16000 RPM, run on a 10% SDS Page gel, and transferred to a polyvinylidene fluoride membrane. The extracellular domain of Notch1 was detected using monoclonal anti-Notch1 antibody (A6, Abcam) and visualized with HRP-conjugated anti-mouse-IgG (Fc) (Sigma Aldrich).

Cell-based Notch-ligand binding assay

Stable HEK 293T cell lines expressing both Lunatic Fringe and wild-type or *O*-fucose mutant 3xFlag-Notch1 were maintained in DMEM containing 10% FBS. One day prior to the experiment, 3×10^6 cells were trypsinized into single cell suspension and replated in serum free medium containing 1% FBS in a 10cm dish. Cells were harvested and washed with FACS staining solution (Hank's Balanced Salt Solution supplemented with 0.5% of albumin, 0.01% NaN_3 and 0.01 g/L of $CaCl_2$). After blocking with the Fc block (eBioscience) for 10 minutes, 2.5×10^5 cells were aliquoted and incubated in 100 µl serum free medium containing either human IgG or various concentrations of soluble Notch ligand, ranging from 1 nM to 32 nM in two-fold increments, for 30 minutes at room temperature. After washing with FACS staining solution, cells were incubated with PE-conjugated anti-human IgG (γ-chain) antibody (Sigma Aldrich) for another 30 minutes on ice. Following two additional washes, these cells were analyzed using a FACS Aria (PerkinElmer). While cells were incubated with soluble Notch ligands, surface expression of Notch1 receptor for each cell line was characterized concurrently. After blocking with Fc block, 2.5×10^5 cells were incubated with biotinylated anti-Flag antibody (M2, Sigma Aldrich) in FACS staining solution for 30 minutes on ice. These cells were then stained with PE-conjugated streptavidin. The analysis was again performed using FACS Aria. Normalized binding affinity of Notch ligand was reported

as a ratio of mean fluorescence intensity for the soluble Notch ligand staining to that for the anti-Flag antibody staining. Each binding experiment was performed three times.

Cell surface expression assay

Bicistronic expressing plasmids were made by cloning full length cDNAs encoding mouse Notch1 *O*-fucose mutants into the multiple cloning site of an EYFP bicistronic expressing vector (Clontech, Mountain View, CA). Surface expression of Notch1 receptors in the absence of individual *O*-fucose site was studied using flow cytometry. Two days prior to the experiment, 5×10^5 HEK cells were plated in 6-well plates and cultured overnight in penicillin/streptomycin-free DMEM medium containing 10% FBS. Next morning, cells were transfected with either wild-type or *O*-fucose mutant 3xFlag-Notch1-EYFP bicistronic expression constructs using lipofectamine 2000. Six hours following the reaction, transfection reagent was replaced with fresh medium containing 1% FBS, in which cells grew for another 18 hours. Cell surface expression of Notch1 was then characterized using monoclonal anti-Flag antibody (M2) and PE-conjugated streptavidin, as described previously. The quantity of 3xFlag-Notch1 surface expression was determined by the mean fluorescence intensity of anti-Flag antibody staining in a given EYFP expression.

Statistical Analysis

Paired two-sided t-test was performed to test differences for continuous variables. The confidence level was set at $p < .05$.

Results

Generation and functional binding of Notch soluble ligands.

Soluble Notch ligands were generated with constructs fusing the entire extracellular domain of each Notch ligand in frame to the human IgG1 Fc region (Figure 4-1a). Production of soluble Dll1, Dll4 and J1 was detected in concentrated supernatants of transiently transfected HEK cells using the anti-human IgG (Fc) antibody by Western blot (Figure 4-1b). Production of soluble J2 was undetectable in these supernatants (data not shown). To generate a large quantity of J2, the supernatant from a stable expressing cell line was collected and concentrated (Figure 4-1b). Running on a reducing gel, both Dll1 and Dll4 were observed to be in the range of 120 to 130 KDa whereas J1 and J2 migrated to a position of approximately 200 KDa. To characterize the activity of these ligands, binding of soluble Dll1 and Dll4 to COS-7 cells, a cell line expressing both Notch receptors and Fringe glycosyltransferases, was analyzed by flow cytometry. Both soluble Dll1 and Dll4 bound to COS-7 cells, with Dll4 staining was more intensely (Figure 4-1c).

3xFlag-epitope on Notch1 does not interfere with normal Notch function

The ability to characterize the cell surface expression of Notch1 receptor is critical to properly evaluate the biological regulation of *O*-fucose exerted on Notch receptors. Currently there are no antibodies to uniquely recognize Notch1 on cell surfaces. In an effort to develop a sensitive and simple assay to detect Notch1 surface expression using a commercially available antibody and flow cytometry, we inserted a 3xFlag epitope at the amino terminus of Notch1 receptor (Figure 4-2a). To determine whether the exogenous epitope interferes with Notch1 receptor activity, both soluble Notch binding and signaling experiments were performed. Flow cytometry analysis of

HEK 293T cells that were transiently transfected with either wild-type or 3xFlag-tagged Notch1 demonstrated that the monoclonal anti-Flag antibody recognized the epitope-tagged but not wild-type Notch1 or control vector (Figure 4-2b). Moreover, soluble Dll4 bound to cells bearing either wild-type or 3xFlag-tagged Notch1 receptors. These findings suggest that the 3xFlag epitope at the N-terminus of Notch1 can be identified by the anti-Flag antibody without compromising the normal synthesis and trafficking of Notch1 protein to the cell surface. To further ensure that the installed exogenous peptide does not interfere with normal Notch activity, an *in vitro* plate-bound ligand-mediated Notch signaling assay was performed. HEK 293T cells that were transiently transfected with either vector control, wild-type, or 3xFlag-tagged Notch1 in addition to Lunatic Fringe, Notch-driven (TP1) *firefly* luciferase reporter and *Renilla* luciferase reporter, were cultured on plate-bound Dll1, Dll4 or human IgG control for 24 hours prior to performing the luciferase assay. The *firefly* luciferase activity was normalized to the *Renilla* luciferase activity and Notch activities were reported as a relative value to the corresponding human IgG control set at 100% (Figure 4-2c). Similar relative Dll-mediated Notch signaling between wild-type and 3xFlag-tagged Notch1 was observed.

Fringe modification of Notch1 receptor enhances immobilized Dll1- and Dll4-mediated Notch signaling but reduces J2-mediated activity

Previous studies have demonstrated that Fringe glycosyltransferases modify *O*-fucose by attaching *N*-acetylglucosamine through a covalent bond and the elongated sugar molecules on Notch receptors modulate Notch signaling. To examine the biologic function of Fringe proteins, the extracellular domain of Notch1 receptor (ECN) was

dissociated and collected from HEK 293T cells that were transiently transfected with either a vector control or a Lunatic Fringe construct in the presence of either wild-type or 3xFlag-tagged Notch1. Western blot analysis indicated that the ECN fragment migrated slower in the presence of Lunatic Fringe (Figure 4-3a), supporting a previous finding that Lunatic Fringe adds additional molecules to the extracellular domain of Notch1. To further evaluate how Notch signaling changes as a function of Fringe modification, immobilized soluble Dll-induced Notch1 activities were compared between Lunatic Fringe transfected cells and vector transfected controls. HEK cells cultured in the presence of Lunatic Fringe yielded a modest but significant increase of the relative Notch-specific luciferase activity by approximately two-fold (Figure 4-3b). Using the same experimental approach, we further demonstrated that Fringe protein homologs differentially enhanced Delta-like ligand-mediated Notch1 signaling (Figure 4-3c). Of the three Fringe homologs, it appeared that Lunatic Fringe produced the strongest positive Notch1 activity. Interestingly, the presence of Lunatic and Manic Fringe reduced J2-mediated Notch1 activity significantly by approximately 40-50% and J1 produced Notch signaling at background levels regardless whether Fringe was present or not (data not shown).

To examine how each Fringe glycosyltransferase regulates ligand-mediated Notch signaling, the binding between soluble ligands and HEK cells expressing 3xFlag-tagged Notch1 plus one of the three Fringe proteins or vector control were analyzed by flow cytometry. Individual cell suspensions were prepared and incubated with 32 nM of soluble Dll1, Dll4 or J2 at room temperature. The binding affinity of a soluble ligand was normalized to the surface Notch1 expression level (ratio of the soluble ligand staining to

the anti-Flag antibody staining). In the absence of Fringe modification, soluble Dll4 bound more readily to Notch1 receptor than soluble Dll1 (Figure 4-3d). Similar to what has been reported in *Drosophila* (120), Fringe modification enhanced the affinity of soluble Delta-like ligands to Notch1 receptor while either has no effect or reducing soluble J2 binding (Figure 4-3d). This finding suggests that Fringe protein regulates Notch signaling, at least in part, by modulating the binding affinity between Notch1 and its ligands.

Mutations of multiple O-fucose sites result in the reduction of immobilized ligand-mediated Notch1 signaling

Our previous results suggested that immobilized Notch ligand was capable of inducing modest Notch signals. To ensure that the observed Notch activity was indeed generated by immobilized ligands, we performed signaling studies in the presence of gamma secretase inhibitor (GSI), a Notch-specific inhibitor (130). When transiently transfected HEK cells were cultured in wells coated with soluble Dll1, Dll4 or J2, addition of 10 μM of GSI inhibited Notch activity by 40-50% when compared to the addition of DMSO (Figure 4-4a). These results confirm that activation of Notch signaling generated using plate-bound ligand is specific and indicate the utility of this assay to assess the biological function of individual *O*-fucose on Notch1 receptors.

We had previously observed that stromal cells expressing Notch ligand Dll1, Dll4 and J2 provided necessary Notch signals to support the differentiation of bone marrow progenitors into mature T cells in culture (data not shown). Here, we examined how each *O*-fucose site on Notch1 regulates Dll1-, Dll4- and J2-mediated Notch activity using the

plate-bound ligand assay described above (Figure 4-4b). Notch1 constructs encoding either wild-type or individual *O*-fucose site mutations were introduced into HEK 293T cells. For Dll-mediated signaling, cells were also cotransfected with the Lunatic Fringe plasmid. When compared with wild-type Notch1, *O*-fucose mutations in EGF9 and EGF12 resulted in a 40% reduction in Dll1- and Dll4-mediated Notch signaling. The mutations in EGF24 and EGF26 resulted in a lower, but still significant reduction in Dll4-derived Notch signaling. The analysis of J2-induced Notch signaling indicated the EGF9 mutant yielded a more significantly lower relative activity while mutation in EGF12 resulted in a slight but not significant decrease in Notch activity. Other *O*-fucose mutations, including EGF8, EGF24, EGF26 and EGF27, did not appear to change immobilized ligand-mediated Notch activity when compared with wild-type. Interestingly, none of the mutants analyzed produced a completely null phenotype, even when Notch1 receptor contained multiple *O*-fucose mutations, including EGF12, 24, 26 and 27.

Ablation of O-fucose sites at different EGF domains differentially reduces Notch1-ligand binding

As we identified multiple *O*-fucose sites critical to the modulation of Notch activity, we were interested in characterizing how these sites modify Notch and ligand interaction. The cell based binding assay was performed with various concentrations of soluble Notch ligand, with Delta-like-ligands and J2 bindings performed in the presence and absence of Lunatic Fringe modification, respectively (representative flow cytometric analysis of soluble Dll1 binding is in Figure 4-5a). The binding was normalized to

surface Notch1 expression to account for the differences in binding sites available in each cell line. We first found that the amount of Dll1, Dll4 and J2 bound to wild-type Notch1-bearing HEK cells increased in proportion to the ligand concentration (Figure 4-5b, c, d and Figure 4-6). While the binding of Dll1 to wild-type Notch1 expressing HEK cells saturated at 16 nM, Dll4 had not reached a plateau at 32 nM. *O*-fucose mutations at both EGF9 and EGF12 resulted in a significant reduction of soluble Dll1 binding (Figure 4-5b). Analysis of the soluble Dll4 binding revealed that EGF12 mutation produced a markedly reduced binding phenotype (by approximately 70%) while ablating the *O*-fucose site in EGF9 did not appear to change the soluble Dll4 affinity to Notch1 receptor (Figure 4-5c). The level of soluble J2 binding was consistently low. Although mutations at multiple *O*-fucose sites appeared to interfere with J2 binding, both mutations in EGF9 and EGF12 resulted in the most significant decrease (Figure 4-5d and Figure 4-6). Together, these findings indicate that multiple *O*-fucose sites participate in the normal binding between Notch1 and its ligands. Furthermore, the reduced Notch signaling that we observed in our previous experiment is partly attributed to the binding defect in the presence of *O*-fucose mutations in EGF9 and EGF12.

Elimination of individual O-fucose sites differentially reduces the surface expression of Notch1 receptors

We postulated that another mechanism by which individual *O*-fucose site may regulate Notch signaling is through the modulation of protein trafficking. To test this we utilized the 3xFlag-tagged Notch1 for the detection of its cell surface expression. HEK cells were transfected with a bicistronic EYFP-expression construct encoding 3xFlag-

tagged Notch1 with or without the individual *O*-fucose mutation. The surface Notch1 of cells that expressed a similar quantity of EYFP fluorescence intensity was measured using the monoclonal anti-Flag antibody. Here, the expression of EYFP served as a surrogate marker for the total expression of Notch1 receptor. A ratio of surface Notch1 to EYFP expression was calculated to account for the difference in total Notch1 expression. In the absence of the Lunatic Fringe modification, the presence of mutations in EGF9 and EGF12 decreased the surface expression level of Notch1 by 60% and 15%, respectively (Figure 4-6a). *O*-fucose sites reside in other EGF domains did not appear to change Notch1 surface distribution significantly. When this experiment was subsequently performed in the Lunatic Fringe expressing HEK cell line, we found that mutations at all EGF domains investigated, with the exception of EGF24, resulted in a statistically significant reduction of Notch1 surface expression (Figure 4-6b).

Discussion

Evidence supporting the proposal that the Notch1 receptor modulates commitment of thymic progenitors to different lineages is controversial. A major problem on solving the role of Notch in thymocyte lineage development is the inability to monitor the expression of Notch1 on the cells. Resolution will require the generation of better tools and assays to monitor the expression. Notch1 molecules are highly conserved among mammalian species, for example, there is a 95% homology between humans and mice. This homology has made it difficult to develop antibodies to recognize Notch1 on the cell surface. Different experimental methods, including *in situ* hybridization (131, 132), protein dissociation assays (129) and biotinylation of cell surface proteins (125)

have been used although each approach has limitations. To circumvent this obstacle, we developed a simple assay to detect surface Notch1 expression using flow cytometry by cloning a 3xFlag epitope at the amino terminus of Notch1 receptor. Both the ligand-mediated Notch1 signaling assay and the soluble Dll4 binding experiment unambiguously showed that epitope-tagged Notch1 functions similarly to its wild-type counterpart. The detection of tagged-Notch1 by a monoclonal anti-Flag antibody further indicated that the installation of an epitope can be efficiently utilized as a tool for the detection of cell surface Notch1.

Previous studies have demonstrated that soluble Notch ligands, including Dll1, J1 and J2 can physically interact with the Notch protein (133). Furthermore, plate-bound soluble Dll1 can activate Notch in both mouse myoblast and human osteosarcoma cell lines (128). These findings suggest that soluble ligands are capable of inducing Notch activity when they are fixed to a hard surface. Here, we cloned cDNAs for all five Notch ligands from a new born mouse thymus and generated soluble ligands by fusing the extracellular domain of each ligand to the nucleotides encoding the human IgG Fc domain in frame. Of the five soluble ligands, Dll3 degraded quickly following its collection (data not shown). Using a cell based binding assay, our results showed that soluble Dll1 and Dll4 bind to COS-7 cells (Figure 4-1c). When these ligands are immobilized to plates, they are capable of inducing Notch1 activity in HEK 293T cells (Figure 4-2b). Together, these results confirm the utility of soluble ligands to study how sugar molecules regulate Notch1-ligand interaction and thus the activation of Notch.

Fringe glycosyltransferases modify Notch receptors by adding *N*-acetylglucosamine to specific *O*-fucose moieties (59). Although it has been well

established that Fringe enhances Dll-mediated Notch signaling while inhibiting Serrate (Jagged)-driven signaling in *Drosophila,* the function of its mammalian counterpart is still not completely understood. To date, there has been only one study that examined how Lunatic, Manic and Radical Fringe modulate Notch activity mediated by Dll1 and J1 (134). In their study, all three Fringe homologs increase Dll1 binding and activation of Notch1. When Lunatic and Manic Fringe suppress J1-mediated Notch1 activity, Radical Fringe unexpectedly enhances the signaling induced by J1. Using the soluble ligand-based experimental approach, we broadened the scope of the prior study by characterizing the modulation of each Fringe homolog on Notch activity mediated by all Notch ligands. Consistent with the previous finding, we found that all three Fringe proteins potentiate Dll1 and Dll4 binding and Notch activity. We further demonstrated that Lunatic and Manic Fringe proteins reduce the binding affinity between J2 and Notch1. This reduction of binding may, at least in part, explain the decrease in J2-mediated Notch1 activity in the presence of these two Fringe proteins. Unlike the other homologs, Radical Fringe does not appear to significantly change J2 binding and Notch1 activity. Interestingly, immobilized J1 does not produce any significant Notch signaling regardless of whether the Fringe protein is present or not. This may stem from the possibility that J1 generates Notch signaling more readily through a different receptor such as the Notch2 receptor (133). Alternatively, immobilized J1 may be incapable of efficiently instigating the necessary proteolytic events to release the intracellular domain of Notch, thus eliciting Notch activity.

Mutational studies have been critical to understanding how *O*-fucose regulates Notch activity. Since *O*-fucose is attached to the hydroxyl group of the serine or

threonine residue, substitution of a serine residue with an alanine eliminates both *O*-fucosylation and Fringe modification. Previous studies have identified that highly conserved *O*-fucose sites on the Notch1 receptor play a critical role in regulating ligand-mediated Notch activity (125). In *Drosophila*, eliminating the *O*-fucose site in EGF12 supports embryonic neurogenesis but not wing boundary formation (135). Mice carrying a targeted *O*-fucose deficiency in EGF12 of the Notch1 receptor produce a mild developmental defect in the T-cell compartment (111). These studies demonstrated that the individual *O*-fucose site in EGF12 alone is not sufficient to account for all the regulation of Notch signaling. Furthermore, the absence of Notch activity and ligand binding in Pofut1-deficient embryonic stem cells supports the notion that multiple *O*-fucose sites contribute to the regulation of Notch activity (97). In this study, we aimed to characterize the role of informative *O*-fucose site in regulating Notch1 activity. We showed that *O*-fucose sites in EGF 9 and 12 are essential in normal Dll1, Dll4 and J2-mediated Notch1 signaling. Furthermore, mutations in EGF24 and EGF26 result in a minor but statistically significant reduction in Dll4-mediated Notch1 activity. In contrast, other *O*-fucose sites including EGF8 and EGF27 do not appear to change ligand-mediated Notch1 activity. Overall, these findings suggest that *O*-fucose sites reside in different EGF domains and differentially contribute to Notch1 signaling.

Mutations of *O*-fucose sites in EGF9 and 12 result in partial, but significant loss of Notch1 activation by Dll1, Dll4 and J2. The reduced Notch1 activity in the EGF12 mutant can be partly attributed to the dysregulation of Notch-ligand binding as this mutation significantly decreases the binding affinity between Notch1 and its soluble ligands (Figure 4-5b-d). This observation is consistent with the previous report that the

EGF12 domain is essential for the ligand interaction (126, 127). Our finding that the *O*-fucose moiety resides in the EGF9 domain, playing an important role in Notch1 induction is somewhat unexpected. Absence of this particular sugar moiety appears to reduce Notch1-ligand binding as well as cell surface expression of Notch1 (Figure 4-5c,d and Figure 4-7). It is possible that removal of the *O*-fucosylation site in EGF9 modifies the structure of the Notch1 receptor. This alteration may compromise Notch1 stability and/or disturb the normal pattern of intracellular protein traffic. When they are localized to the cell surface, these Notch1 receptors may not be capable of interacting with ligands properly. Alternatively, these altered Notch1 receptors may turnover rapidly while on the cell surface which leads to a relatively lower expression at any given time. In contrast, the EGF24, 26 and 27 domains are localized in the *Abruptex* region of Notch1, in which missense mutations have been reported to enhance Notch activity in *Drosophila* (136). Here, we show that the *O*-fucose moieties in these domains do not appear to regulate Dll1- and J2-mediated Notch1 activity. However, absence of these sugar molecules in EGF24 and 26 results in a mild but statistically significant reduction of Notch1 activity induced by Dll4. This phenotype is likely due to a loss of cell surface expression of Notch1 in the presence of Lunatic Fringe (Figure 4-7b). This observation is consistent with the previously published finding that aberrant Fringe modification of the human Notch3 receptor results in the abnormal accumulation of Notch3 in intracellular compartments (137). Overall, our finding suggests that *O*-fucose moieties at different location contribute to Notch activity via different regulatory mechanisms. It is a series of these intricate regulatory mechanisms that regulate the outcome of Notch signal in a dose dependent manner.

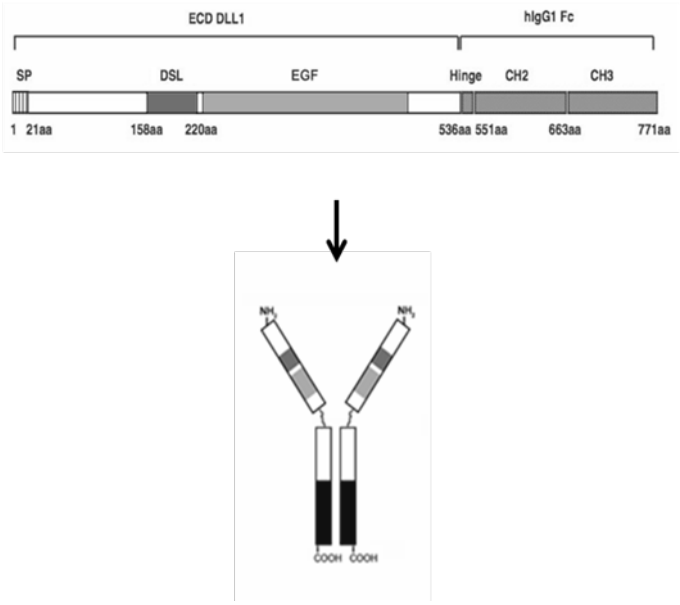
Notes to Chapter IV

I want to thank Bronislawa Petryniak for performing the western blot analysis, Notch dissociation assay and establishing the cell-based binding assay conditions. I also want to express my appreciation to Dr. Jeongsup Shim for the generation of soluble Notch ligands. I am indebted to both Dr. John Lowe and Dr. Robert Fairchild for their constructive criticisms and recommendations regarding the preparation of the manuscript. DNA plasmids containing soluble Notch ligands and Fringe glycosyltransferases were made by Bronislawa Petrinyak. 3xFlag-tagged mouse Notch1 cDNA was made by Dr. Jeongsup Shim.

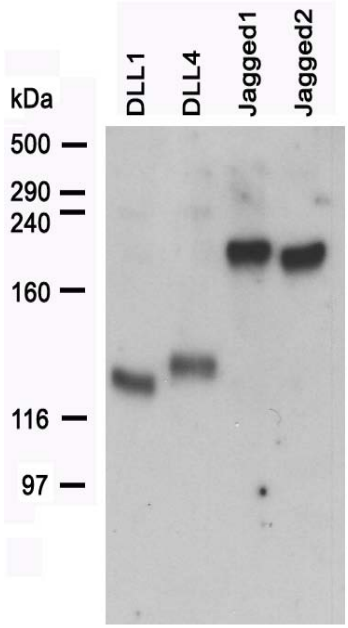
Figure 4-1. Generation and characterization of soluble Notch ligands. a. Schematic representation of soluble Dll1 containing the signal peptide (SP), the DSL domain, EGF-like repeats and the human IgG1 Fc region, including the hinge, CH2 and CH3 domains. All other soluble Notch ligands were constructed in the same fashion. **b.** Western blot analysis of soluble Dll1, Dll4, J1 and J2. The blot was probed using HRP-conjugated anti-human IgG (Fc) antibody. **c.** Representative flow cytometric analysis of COS-7 cells stained with human IgG, soluble Dll1 and soluble Dll4.

Figure 4-1

a.


ECD DLL1
hIgG1 Fc
SP
DSL
EGF
Hinge
CH2
CH3
1
21aa
158aa
220aa
536aa
551aa
663aa
771aa
NH2
NH2
COOH
COOH

b.


kDa
500
290
240
160
116
97
DLL1
DLL4
Jagged1
Jagged2

c.

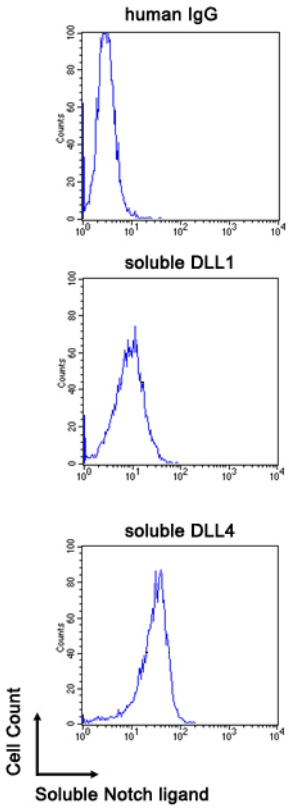
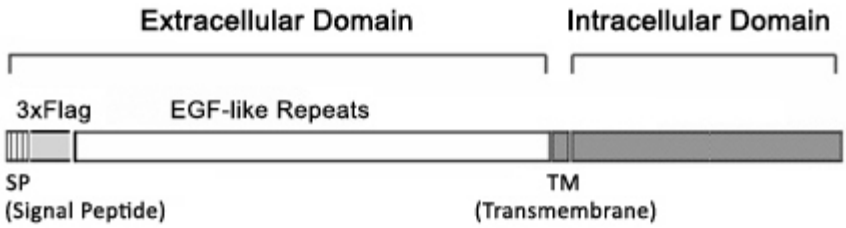

human IgG
soluble DLL1
soluble DLL4
Counts
Cell Count
Soluble Notch ligand

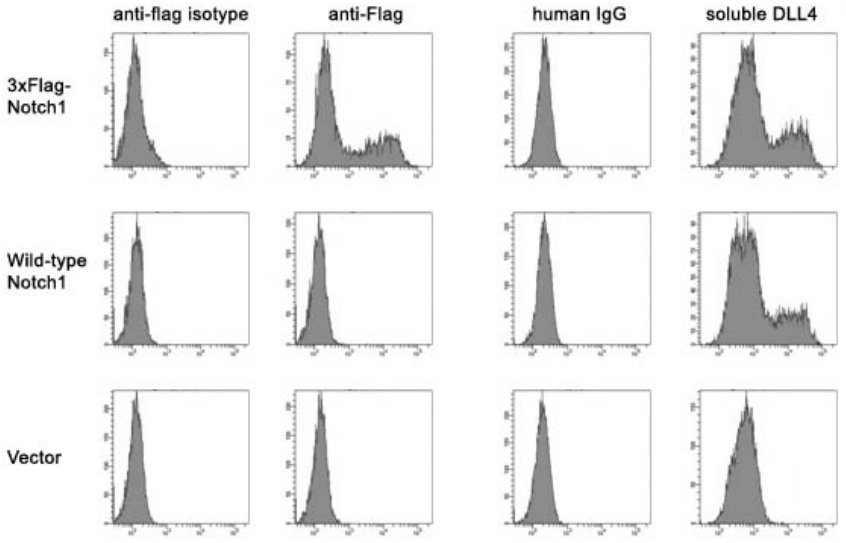
Figure 4-2. An NH2-terminal 3xFLAG epitope appended to mNotch1 does not disrupt binding of Dll1 to mNotch1, nor does it disrupt mNotch1 signaling by Dll1 or Dll4. a. Schematic representation of the Notch1 protein containing a 3xFLAG epitope at its amino terminus. **b.** Staining of HEK 293T cells transiently expressing 3xFLAG-tagged Notch1, wild-type Notch1 or an empty vector control (pCDNA3.1), using a monoclonal anti-FLAG antibody, an isotype-matched negative control antibody, a Dll4-IgG fusion protein, or a human IgG negative control (see Materials and Methods). Each such transfection also included co-transfected plasmids corresponding to a Lunatic Fringe expression vector. **c.** *in vitro* plate-bound ligand-mediated Notch signaling assay (see Materials and Methods). HEK 293T cells were transiently transfected with a 3xFLAG-mNotch1 expression vector, or a with wild-type mNotch1 expression vector or with an empty control vector, (pCDNA3.1), Each such transfection also included co-transfected plasmids corresponding to a Lunatic Fringe expression vector, a TP1-luciferase plasmid that reports Notch1 signaling, and a *Renilla* luciferase reporter to allow for control for transfection efficiency. After culturing these cells on plate-bound Dll1-IgG, Dll4-IgG or human IgG, cells were harvested for luciferase activity measurements. Relative Notch1 activity is reported as a ratio of luciferase activity induced by Delta-like ligands over that observed on plates coated with human IgG, normalized to *Renilla* luciferase activity. Relative Notch activities are presented as means ± standard deviations (SD).

Figure 4-2

a.


Extracellular Domain
Intracellular Domain
3xFlag
EGF-like Repeats
SP
(Signal Peptide)
TM
(Transmembrane)

b.


anti-flag isotype
anti-Flag
human IgG
soluble DLL4
3xFlag-Notch1
Wild-type Notch1
Vector

c.

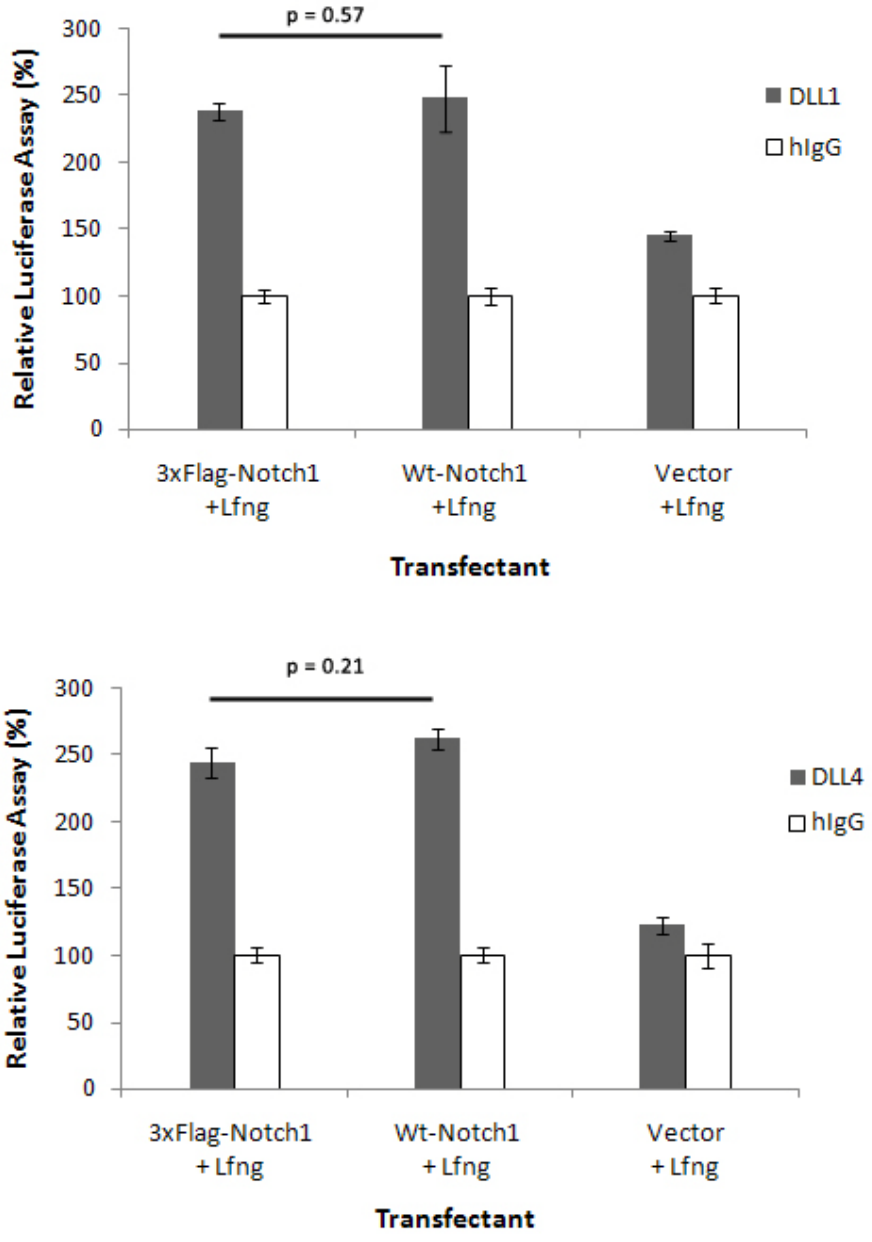
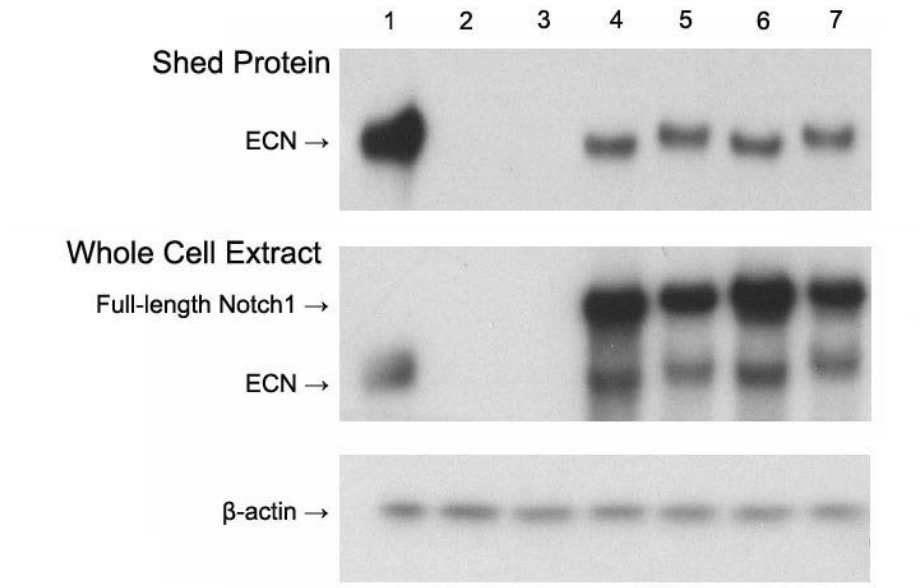

p = 0.57
300
250
200
150
100
50
0
Relative Luciferase Assay (%)
DLL1
hIgG
3xFlag-Notch1 +Lfng
Wt-Notch1 +Lfng
Vector +Lfng
Transfectant
p = 0.21
300
250
200
150
100
50
0
Relative Luciferase Assay (%)
DLL4
hIgG
3xFlag-Notch1 + Lfng
Wt-Notch1 + Lfng
Vector + Lfng
Transfectant

Figure 4-3. Fringe glycosyltransferases modulate mNotch1-Notch ligand interactions and mNotch1-selicited signaling in a ligand-specific manner.
a. Western blot analysis of EDTA-induced dissociation of the Notch1 extracellular domain. HEK 293T cells were transfected with either 3xFlag-Notch1, wild-type Notch1 or vector plasmid, along with either Lunatic Fringe or vector plasmid. The extracellular domain of Notch1 was dissociated using 5 mM of EDTA and analyzed by Western blot using anti-mouse Notch1 antibody (A6). **b.** Dll1- and Dll4-mediated Notch signaling assay in HEK 293T cells expressing either 3xFlag-Notch1 or vector, along with either Lunatic Fringe or vector, TP1 luciferase reporter and *Renilla* luciferase reporter. Relative Notch activity is expressed as a ratio of normalized luciferase activity induced by Delta-like ligands over that obtained by human IgG.
c. Notch activity analysis of HEK 293T cells that were cotransfected with 3xFLAG-mNotch1, TP-1 luciferase reporter, *Renilla* luciferase reporter and either vector or one of the three Fringe homologs (Lunatic Fringe, Manic Fringe or Radical Fringe). Notch signaling was induced by immobilized Dll1, Dll4 and J2; plate bound human IgG serves as the control. Notch activity is reported as a relative activation of normalized luciferase activity induced by Notch ligands over normalized luciferase activity induced by human IgG. 3xFlag-N1: 3xFlag-tagged Notch1
d. Flow cytometry assessment of Fringe-dependent binding of Notch ligands to mNotch1. HEK 293T cells stably expressing 3xFlag-Notch1 and Lunatic Fringe, Manic Fringe or Radical Fringe expression vectors or an empty control vector were incubated with Dll1-IgG, Dll4-IgG or J2-IgG, at concentrations of 32 nM, and were then subjected to flow cytometry analysis (see Materials and Methods). Binding of each Notch ligand to the target cell line is reported as a ratio of mean fluorescence intensity (MFI) obtained for the Notch ligand-IgG chimera staining, relative to the MFI obtained with anti-Flag antibody staining. The data are the averages of the ratios ± SD derived from 3 independent experiments.

Figure 4-3

a.

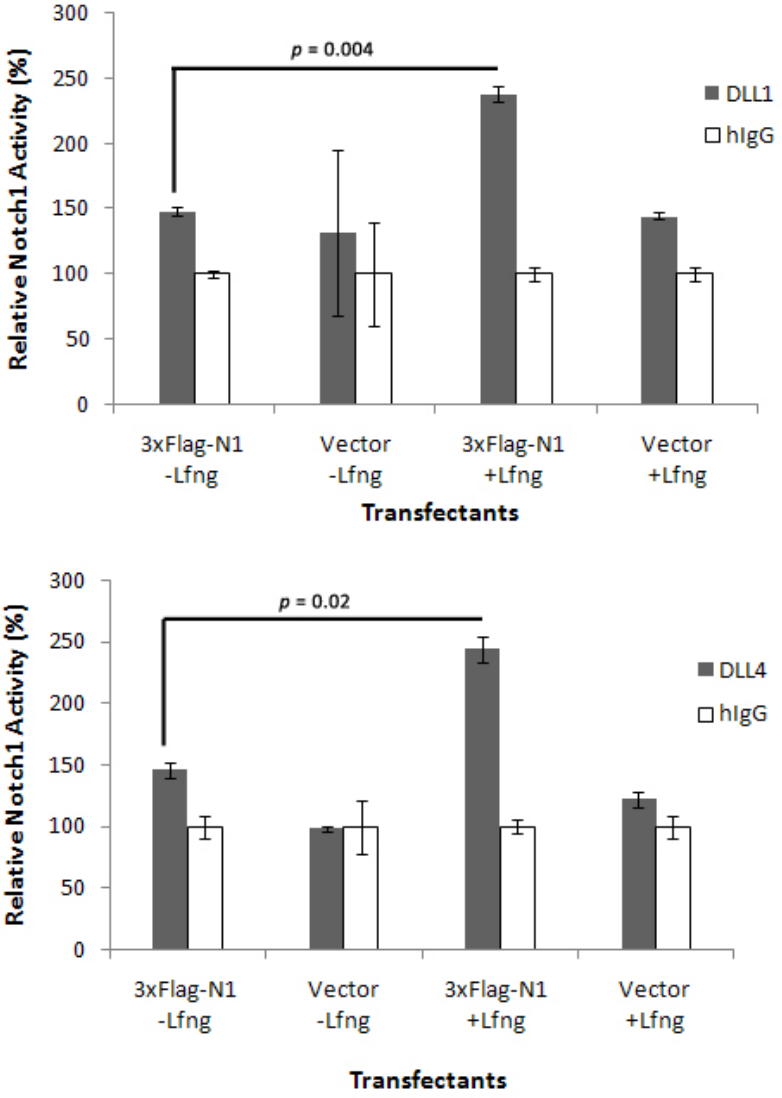


Lane 1: 3xF-mNotch1 from Lec13

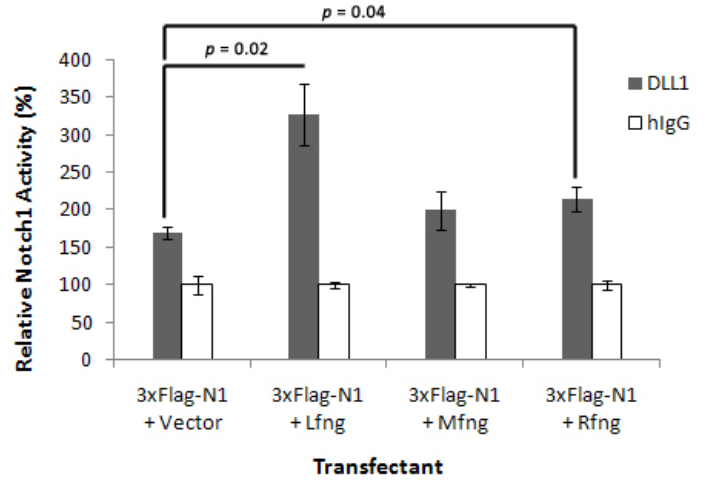
Lane 2:pCDNA3.1 + pZeo Lane 3:pCDNA3.1 + Lfng Lane 4:wt-mNotch1 + pZeo

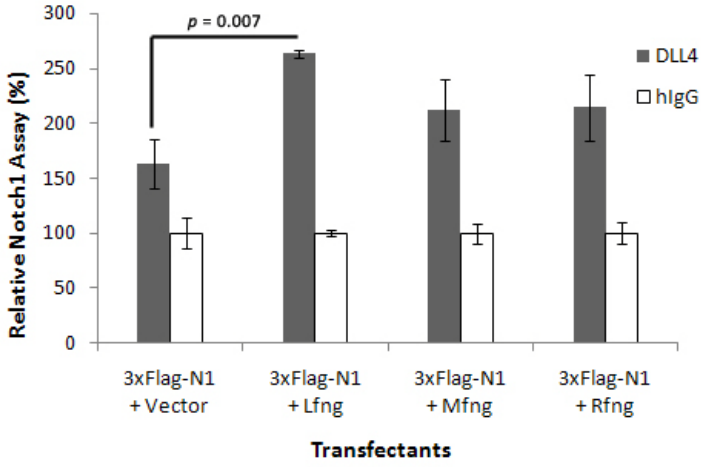
Lane 5:wt-mNotch1 + Lfng Lane 6:3xF-mNotch1 + pZeo Lane 7:3xF-mNotch1 + Lfng

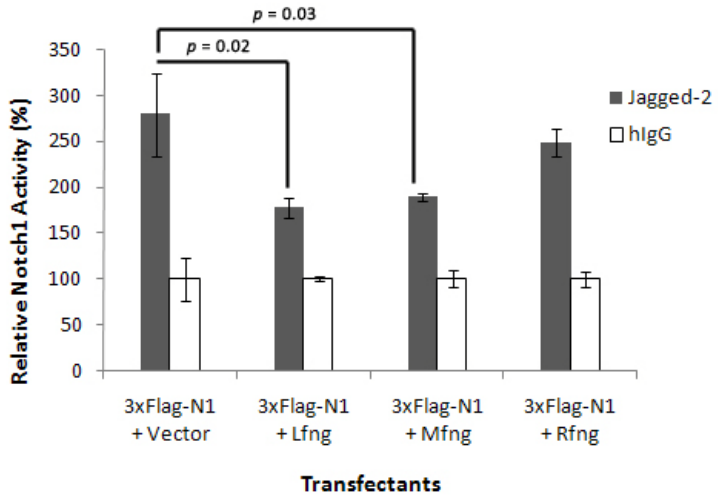
b.


300
250
200
150
100
50
0
Relative Notch1 Activity (%)
p = 0.004
DLL1
hIgG
3xFlag-N1 -Lfng
Vector -Lfng
3xFlag-N1 +Lfng
Vector +Lfng
Transfectants
300
250
200
150
100
50
0
Relative Notch1 Activity (%)
p = 0.02
DLL4
hIgG
3xFlag-N1 -Lfng
Vector -Lfng
3xFlag-N1 +Lfng
Vector +Lfng
Transfectants

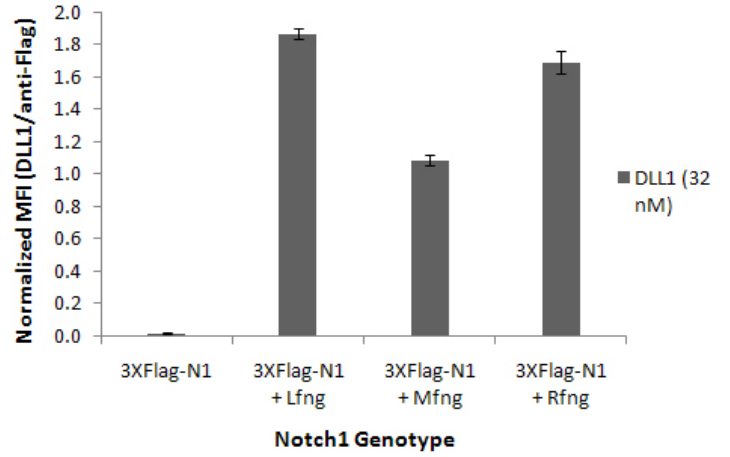
c.

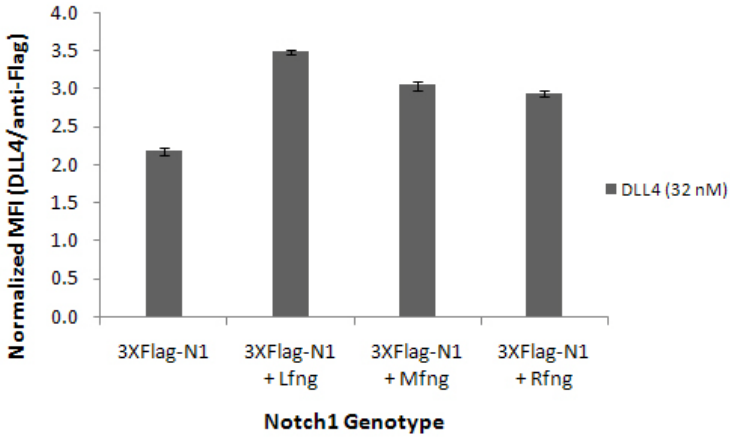

p = 0.04
p = 0.02
400
350
300
250
200
150
100
50
0
Relative Notch1 Activity (%)
DLL1
hIgG
3xFlag-N1 + Vector
3xFlag-N1 + Lfng
3xFlag-N1 + Mfng
3xFlag-N1 + Rfng
Transfectant


p = 0.007
300
250
200
150
100
50
0
Relative Notch1 Assay (%)
DLL4
hIgG
3xFlag-N1 + Vector
3xFlag-N1 + Lfng
3xFlag-N1 + Mfng
3xFlag-N1 + Rfng
Transfectants


p = 0.03
p = 0.02
350
300
250
200
150
100
50
0
Relative Notch1 Activity (%)
Jagged-2
hIgG
3xFlag-N1 + Vector
3xFlag-N1 + Lfng
3xFlag-N1 + Mfng
3xFlag-N1 + Rfng
Transfectants

d.


Normalized MFI (DLL1/anti-Flag)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3XFlag-N1
3XFlag-N1 + Lfng
3XFlag-N1 + Mfng
3XFlag-N1 + Rfng
Notch1 Genotype
DLL1 (32 nM)


Normalized MFI (DLL4/anti-Flag)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3XFlag-N1
3XFlag-N1 + Lfng
3XFlag-N1 + Mfng
3XFlag-N1 + Rfng
Notch1 Genotype
DLL4 (32 nM)

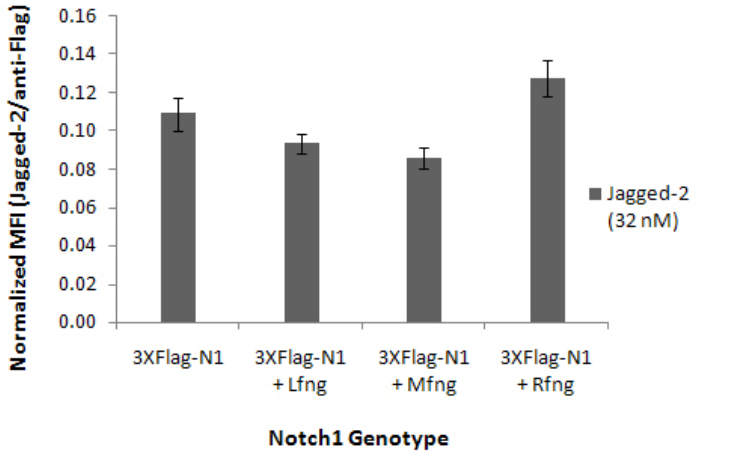
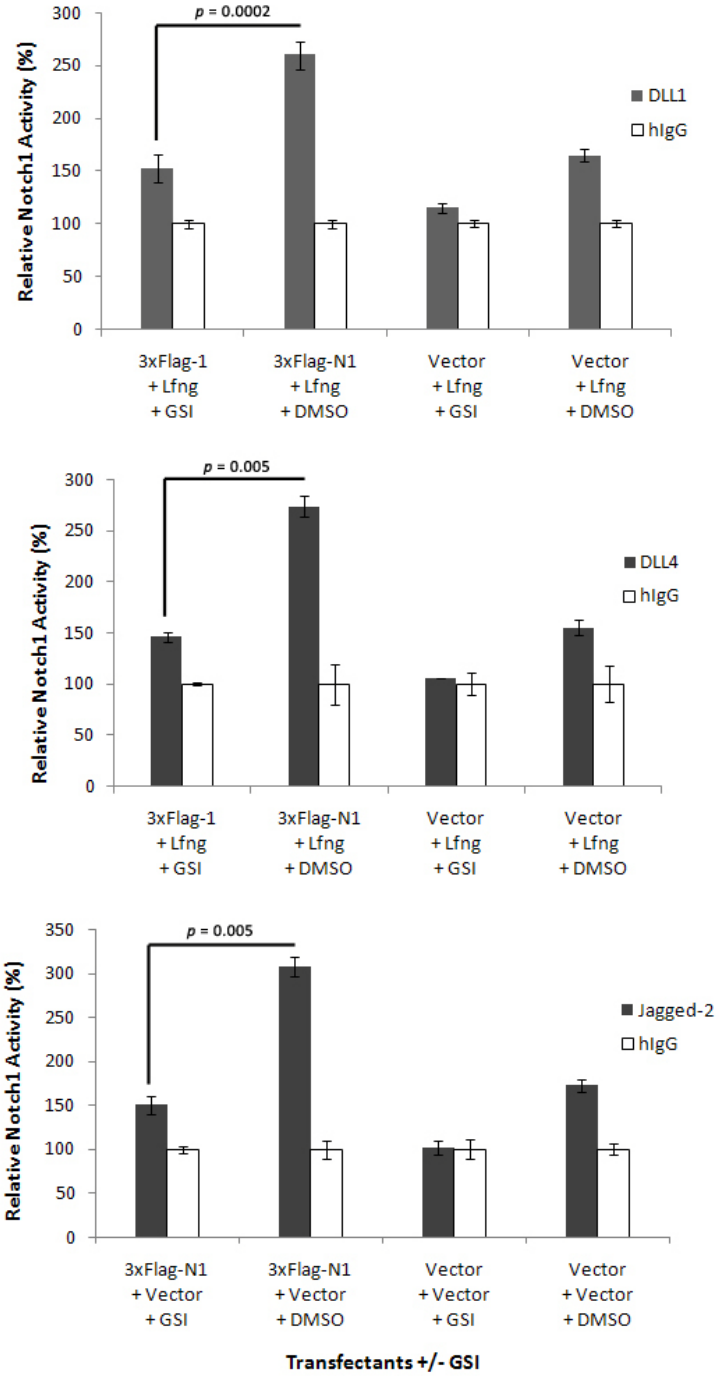

Normalized MFI (Jagged-2/anti-Flag)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
3XFlag-N1
3XFlag-N1 + Lfng
3XFlag-N1 + Mfng
3XFlag-N1 + Rfng
Notch1 Genotype
Jagged-2 (32 nM)

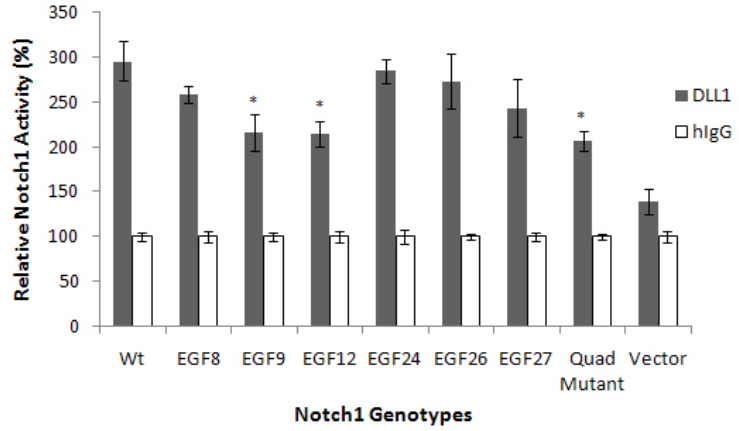
Figure 4-4. Multiple *O*-fucose sites are involved in Notch signaling mediated by immobilized soluble Notch ligands. a. Plate-bound ligand-mediated Notch activity in the presence of Gamma secretase inhibitor (GSI). HEK 293T cells cotransfected with Lunatic Fringe, TP1 luciferase reporter, *Renilla* luciferase reporter and either 3xFlag-Notch1 or vector were cultured in Dll1- and Dll4-coated plates in the presence of GSI or DMSO (control). Same experimental approach is applied to J2-coated plates except HEK 293T cells were transfected with vector plasmid instead of Lunatic Fringe. Relative Notch activity is reported as a ratio of normalized luciferase activity produced by bound ligand over that induced by human IgG. **b.** Relative Notch1 activity induced by immobilized Dll1, Dll4 and J2 in the presence of *O*-fucose mutations. In addition to TP1 luciferase and *Renilla* luciferase reporter constructs, HEK 293T cells were cotransfected with either vector, wild-type or *O*-fucose mutation bearing 3xFlag-Notch1 plasmids. Quad mutant carries a combination of *O*-fucose mutations in EGF12, EGF24, EGF26 and EGF27. Notch1 activity is reported as a relative change of normalized luciferase activity induced by plate-bound soluble ligands over that produced by human IgG. Each experiment was performed in duplicate. Three independent experiments were performed. (* Relative Notch1 activity is significantly lower than that produced by wild-type Notch1, *p*-value is set at less than .05).

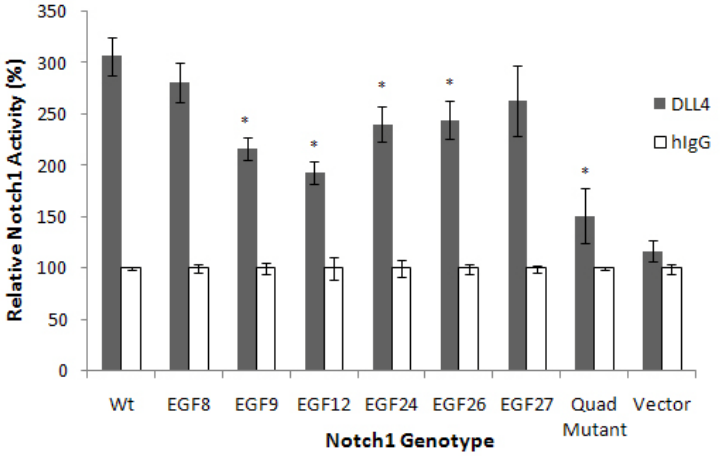
Figure 4-4

a.


p = 0.0002
Relative Notch1 Activity (%)
300
250
200
150
100
50
0
DLL1
hIgG
3xFlag-1 + Lfng + GSI
3xFlag-N1 + Lfng + DMSO
Vector + Lfng + GSI
Vector + Lfng + DMSO
p = 0.005
DLL4
hIgG
p = 0.005
350
Jagged-2
hIgG
3xFlag-N1 + Vector + GSI
3xFlag-N1 + Vector + DMSO
Vector + Vector + GSI
Vector + Vector + DMSO
Transfectants +/- GSI

b.


Relative Notch1 Activity (%)
350
300
250
200
150
100
50
0
DLL1
hIgG
Wt
EGF8
EGF9
EGF12
EGF24
EGF26
EGF27
Quad Mutant
Vector
Notch1 Genotypes


Relative Notch1 Activity (%)
350
300
250
200
150
100
50
0
DLL4
hIgG
Wt
EGF8
EGF9
EGF12
EGF24
EGF26
EGF27
Quad Mutant
Vector
Notch1 Genotype

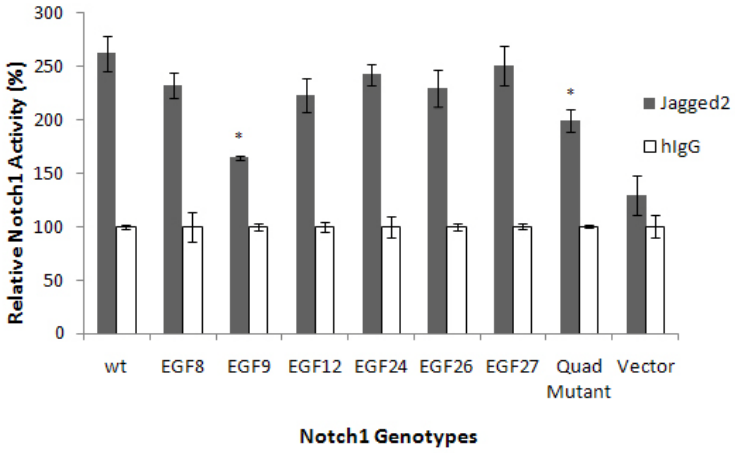
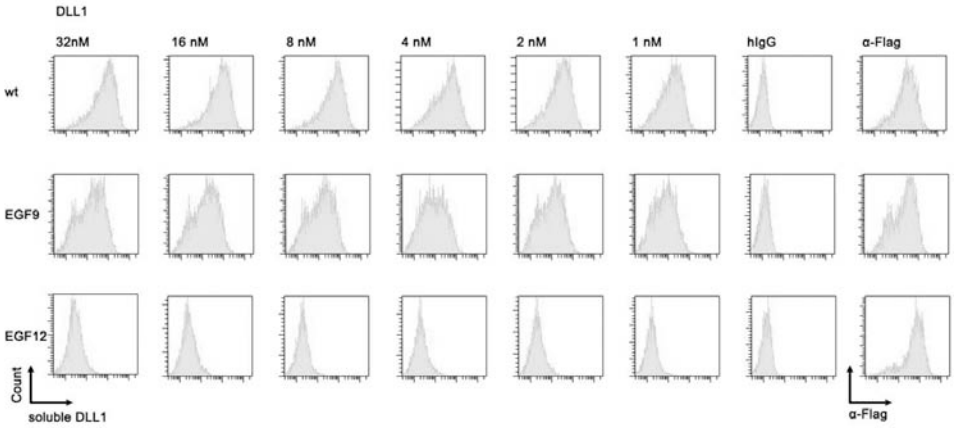

Relative Notch1 Activity (%)
300
250
200
150
100
50
0
Jagged2
hIgG
wt
EGF8
EGF9
EGF12
EGF24
EGF26
EGF27
Quad Mutant
Vector
Notch1 Genotypes

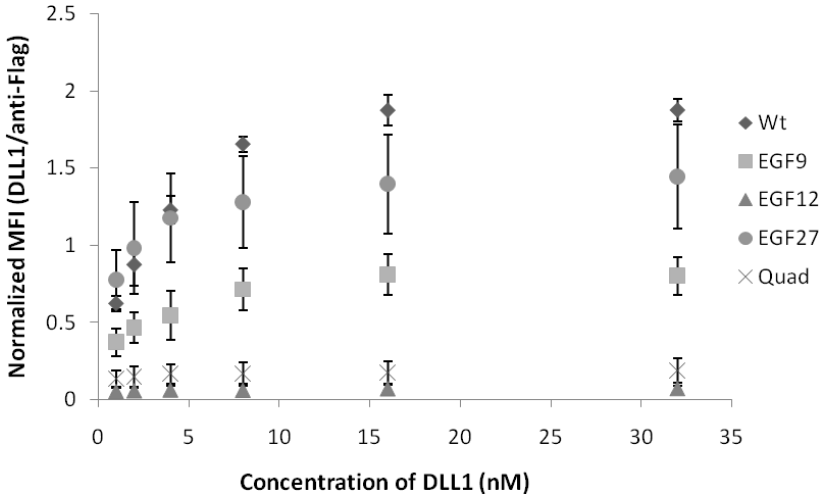
Figure 4-5. Ablation of individual *O*-fucose sites differentially reduces binding of soluble Notch ligand. **a.** Representative flow cytometric analysis of HEK 293T cells expressing 3xFlag-Notch1 bearing individual *O*-fucose mutations EGF9 and EGF12 stained with soluble Dll1 at a concentration ranging from 1 nM to 32 nM (human IgG staining serves as a binding control) and anti-Flag antibody. **b-d.** Binding curves of soluble Dll1 (B), Dll4 (C) and J2 (D) to HEK 293T cells expressing 3xFlag-Notch1 bearing *O*-fucose mutations in EGF9, EGF12, EGF27 and Quad (quadruple mutations including EGF12, EGF24, EGF26 and EGF27). The concentrations for each soluble ligand range from 1 nM to 32 nM in two-fold increments. Soluble Delta-like ligand binding was performed in the presence of Lunatic Fringe. The binding is presented as a ratio of soluble ligand staining over the anti-Flag antibody staining to account for the differences in Notch1 expression in each cell line (results of three independent experiments).

Figure 4-5

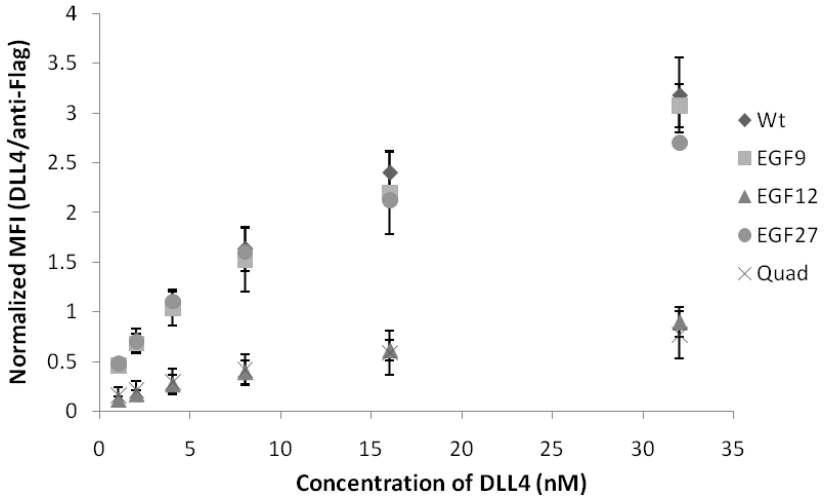
a.


DLL1
32nM
16 nM
8 nM
4 nM
2 nM
1 nM
hIgG
α-Flag
wt
EGF9
EGF12
Count
soluble DLL1
α-Flag

b.


Normalized MFI (DLL1/anti-Flag)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
Concentration of DLL1 (nM)
Wt
EGF9
EGF12
EGF27
Quad

c.


Normalized MFI (DLL4/anti-Flag)
Concentration of DLL4 (nM)
Wt
EGF9
EGF12
EGF27
Quad

d.

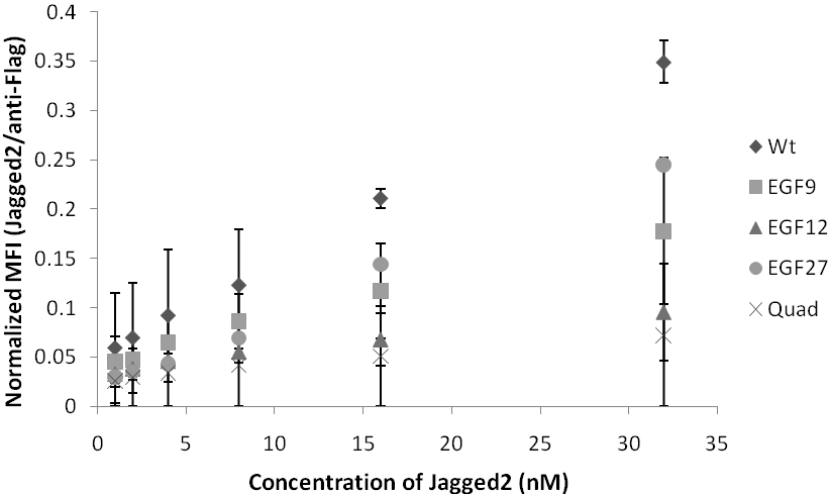

Normalized MFI (Jagged2/anti-Flag)
Concentration of Jagged2 (nM)
Wt
EGF9
EGF12
EGF27
Quad

Figure 4-6. Ablation of individual *O*-fucose mutation differentially reduces binding of soluble Notch ligand. Binding curves of soluble Dll1, Dll4 and Jagged2 to HEK 293T cells expressing 3xFlag-Notch carrying individual *O*-fucose mutations, including EGF8, EGF24 and EGF26. The concentrations of each ligand range from 1 nM to 32 nM in two-fold increments. The binding is reported as a ratio of soluble ligand staining over the anti-Flag antibody staining (results of three independent experiments).

Figure 4-6

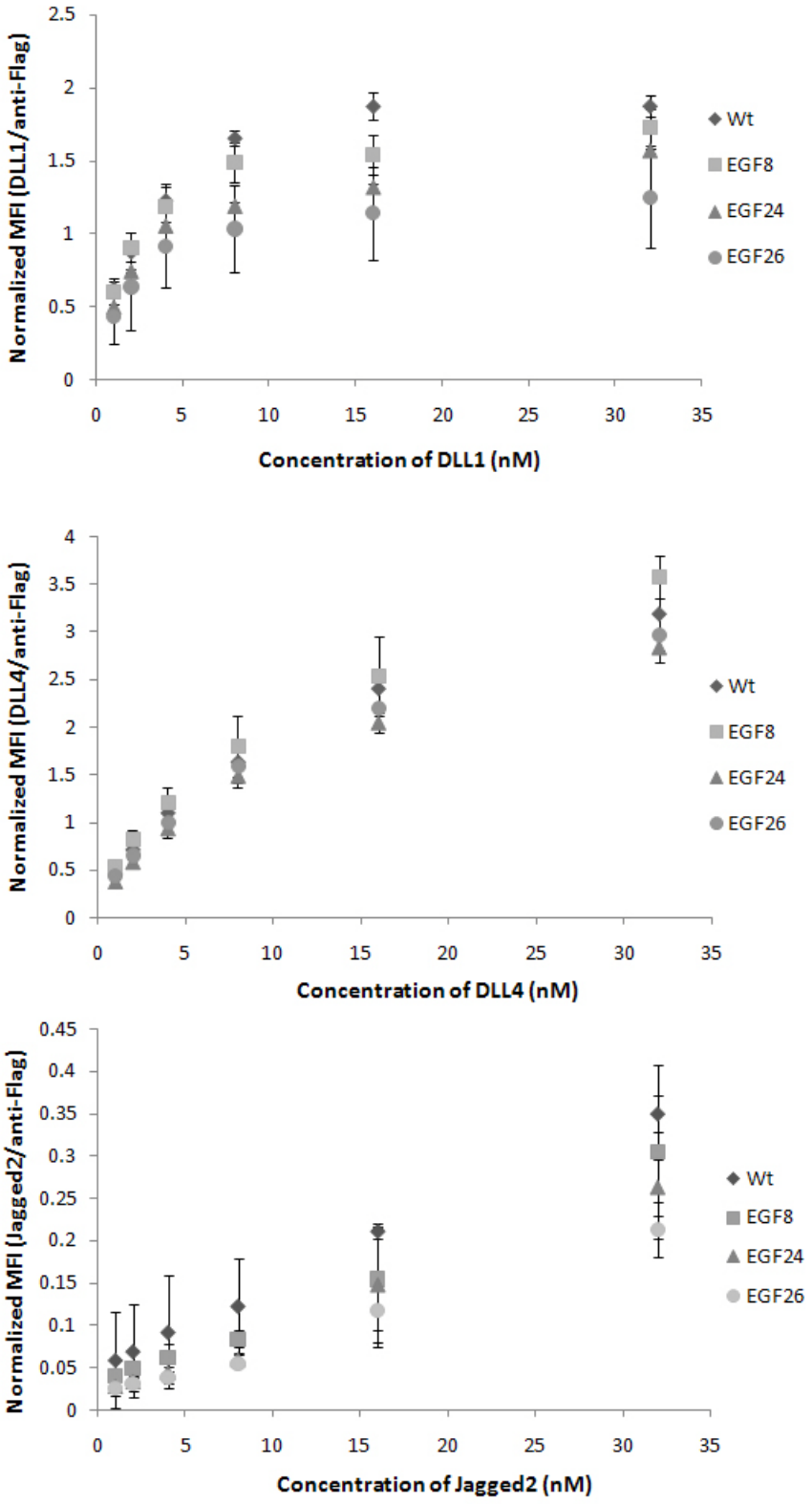
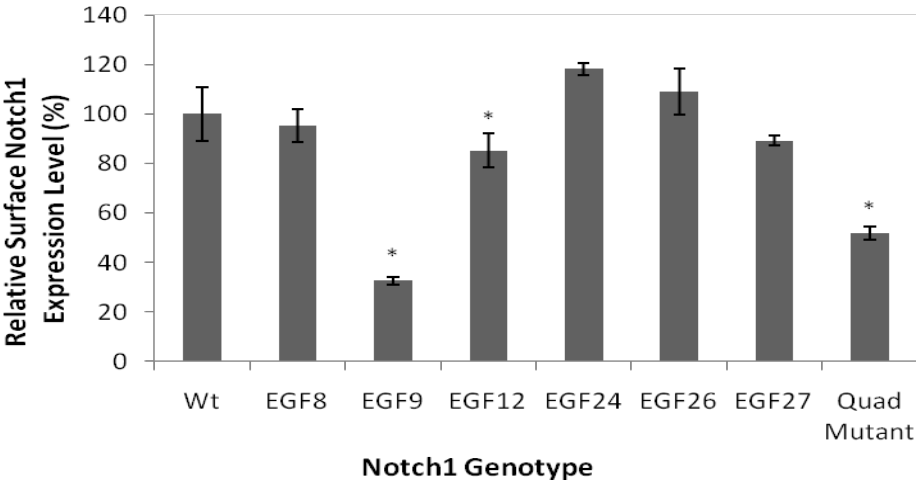

Normalized MFI (DLL1/anti-Flag)
Concentration of DLL1 (nM)
Wt
EGF8
EGF24
EGF26
Normalized MFI (DLL4/anti-Flag)
Concentration of DLL4 (nM)
Wt
EGF8
EGF24
EGF26
Normalized MFI (Jagged2/anti-Flag)
Concentration of Jagged2 (nM)
Wt
EGF8
EGF24
EGF26

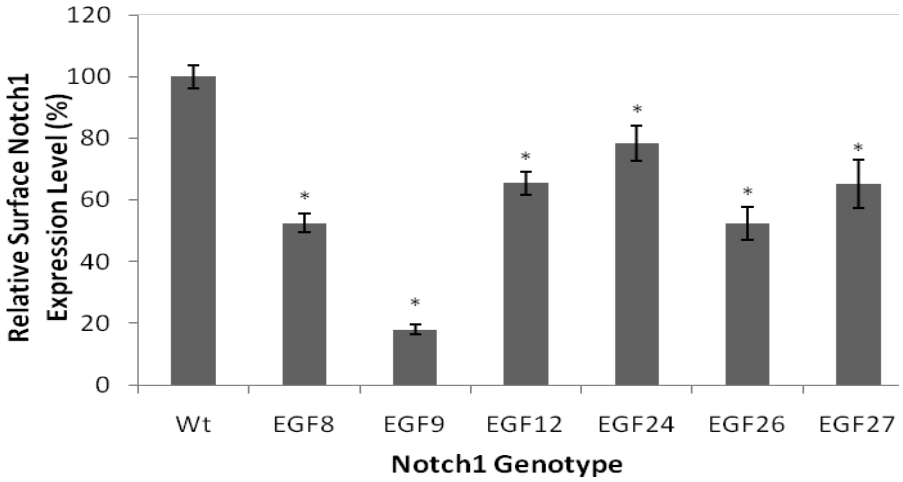
Figure 4-7. Loss of individual *O*-fucose reduces the cell surface expression of Notch1 in the presence of Lunatic Fringe modification. a, b. Cell surface expression of 3xFlag-Notch1 bearing *O*-fucose mutation in the presence **(a)** or absence **(b)** of Lunatic Fringe was quantified using flow cytometry. HEK 293T cells were transfected with bicistronic constructs expressing both EYFP and 3xFlag-Notch1 bearing individual *O*-fucose mutations. The quantity of cell surface Notch1 expression in EYFP-positive cells is calculated as a ratio of the mean fluorescence intensity (MFI) of anti-Flag antibody to that of EYFP expression. The relative surface expression of mutant Notch1 is normalized to that of the wild type Notch1, which is normalized to 100%. Data are representative of three independent experiments, each of which was performed in triplicate.

Figure 4-7

a.


140
120
100
80
60
40
20
0
Relative Surface Notch1 Expression Level (%)
*
*
*
*
Wt
EGF8
EGF9
EGF12
EGF24
EGF26
EGF27
Quad Mutant
Notch1 Genotype

b.


120
100
80
60
40
20
0
Relative Surface Notch1 Expression Level (%)
*
*
*
*
*
*
Wt
EGF8
EGF9
EGF12
EGF24
EGF26
EGF27
Notch1 Genotype

CHAPTER V

Characterizing the Cell Surface Expression of Murine Notch1 Receptors

Abstract

The role of Notch1 in the T versus B lineage decision has been well documented. Its function in the subsequent lineage differentiation stages, however, is still unclear. This ambiguity is partly due to the difficulty in characterizing the surface expression of Notch1 in T cell subsets. As previously mentioned, Notch1 molecules have a high degree of amino acid sequence conservation among mammalian species, which has made it difficult to develop antibodies to detect Notch1 on the cell surface. Several studies have used different experimental methods to circumvent this obstacle although each approach has its limitations. In an effort to determine the stages of T lymphopoiesis where Notch1 is available to activate downstream signaling events, we generated an animal model whose Notch1 is tagged with the 3xFlag epitope at the amino terminus. Mice carrying homozygous 3xFlag-Notch1 alleles did not exhibit any visible developmental defects. In breeding of male and female heterozygotes, the ratio of wild-type/heterozygote/homozygote genotypes in progeny roughly followed the 1:2:1 Mendelian distribution. The up-regulation of the Notch1 surface expression in antibody-activated naïve $CD4^+$ T cells purified from the adult 3xFlag-Notch1 homozygote was determined using a monoclonal antibody against the flag epitope. Together, the installment of the 3xFlag epitope in Notch1 does not interfere with normal mouse

development and reproduction and thus provides a new approach to characterize and quantify cell-type specific expression patterns of the Notch1 receptor.

Introduction

Notch1 activity is critical to the success of T lineage commitment. The site of mature T cell generation is in the thymus. Upon release from the bone marrow, thymocyte progenitors enter the thymus through the cortico-medullary junction, at which the engagement between early thymic progenitors (ETPs), a subset of the thymocyte progenitors defined as Lin^{-} c-Kit^{hi} $CD44^{+}$ $CD25^{-}$ $CD24^{lo}$, and Notch ligands leads to T lineage specification (138, 139). In the absence of either Notch1 or Notch effector RBP-J, mature T cell population is markedly reduced in mice (44, 45). Though the role of Notch1 activity in the T lineage commitment has been well documented, an important question remains unanswered, however: what is the Notch1 regulation at different stages of lineage decision during T lymphoid development?

After initial T lineage commitment, premature thymocytes undergo multiple lineage specifications. Although Notch1 has been implicated in these lineage decisions, evidence supporting the notion that Notch1 modulates αβ versus γδ, CD4 versus CD8 and Th1 versus Th2 lineage commitments is still controversial. Various studies using different animal models have reached conflicting results. Washburn and colleagues showed that $Notch1^{+/-}$ bone marrow progenitors preferentially differentiated into the γδ lineage in chimeric mice whose bone marrows were reconstituted with both $Notch1^{+/+}$ and $Notch1^{+/-}$ bone marrow cells (117). In other complementary studies, Wolfer and colleagues demonstrated that Lck-Cre-mediated loss of function in Notch1 signaling

resulted in the depletion of the αβ lineage T cells while elimination of Notch-dependent transcription factor, CSL, at a later stage gave rise to the defective αβ lineage development (140, 141). On the contrary, adult animals that carry the homozygous Jagged-2-null allele produced a phenotype where the numbers of αβ T cells were normal (142). Moreover, decrease in Notch activity appears to be required for human αβ lineage differentiation (143). It is unclear how these findings can be reconciled although the difference may be attributed to the expression of other Notch ligands in the thymic stroma for the former and the difference in the role of Notch activity between human and mice for the latter. The role of Notch1 in regulating the lineage decision between CD4 and CD8 is also unclear. In previous studies, the adult mice whose Notch signal is constitutively activated had an increase in the CD8 T cell compartment whereas the number of their CD4 T cells was reduced (144, 145). Wolfer and colleagues did not reach the same conclusion in their study, however. Using the TCR promoter-mediated Notch1 knockout mice, they observed that the development of the $CD4^+$ and $CD8^+$ mature thymocytes was undisturbed (146). Similar finding was reported in mice maintaining a targeted CD4-promoter driven deficiency in the CSL locus (141). This suggested that Notch1-mediated signal is dispensable in regulating $CD4^+$ versus $CD8^+$ differentiation. Together, the physiologic role of Notch1 in the immature T lymphoid lineage decisions is still unclear.

In the periphery Notch activity has been implicated in facilitating TCR-mediated T cell activation. The expression of all Notch homologs and the intracellular domain of Notch molecules, an active form of Notch, were up-regulated in activated $CD4^+$ cells (147, 148). In these studies, the productions of IL-2 and IFNγ were markedly reduced

when Notch activation in peripheral T cells was inhibited using gamma secretase inhibitor (GSI). These findings were challenged by the data demonstrating that conditional Notch1-deficiency under the control of CD4-Cre failed to produce any measurable defect in T cell activation (149). Moreover, T cell activation was blocked in cells stimulated with plate bound anti-Notch1, anti-CD3 and anti-CD28 (150). This inhibition was reversed with the treatment of GSI. Although this discrepancy still remains to be resolved, the role of Notch in peripheral T cell activation may be dependent on the cellular context. In yet another binary decision point, the role of Notch activity in the differentiation of naïve $CD4^+$ T cells into Th1 or Th2 lineages is still unclear. Previous studies showed that stimulation of naïve $CD4^+$ T cells with either soluble Dll1-Fc fusion protein or antigen presenting cells (APCs) engineered to express Dll1 gave rise to Th1 polarization (151, 152). In contrast, mice maintaining the targeted deficiency in the CSL locus showed either an enhanced IFNγ production or no observable Th1 versus Th2 defect (141). Similar to the controversies in the immature T subset specification, the role of Notch pathway in the peripheral T cell activity and differentiation still remains to be uncovered.

Difficulties in characterizing the functional role of Notch1 in T lymphoid subset may be partly attributed to the undefined surface expression pattern of Notch1 during the development of thymocytes and to the difficulty in sorting out the activity among Notch homologues (Notch1-3) during thymopoiesis. Notch1 molecules are highly conserved among mammalian species, for example there is a 95% homology between human and mouse. This resemblance has made it difficult to develop antibodies to uniquely

recognize Notch1 on the cell surface. Several studies have used different experimental methods to circumvent this obstacle although each approach has its limitations.

Techniques used to characterize the expression of the mRNA level such as RT-PCR (153-156) and *in situ* hybridization (131, 132) have been commonly employed to generalize the expression of Notch1. This approach may not, however, accurately represent the expression of the Notch1 protein since several levels of post-transcriptional regulations are in place. These include glycan addition, hetero-dimer formation and protein trafficking. Other methods use a direct protein quantifying assay to describe the surface expression of Notch1 in the *in vitro* setting. Rand and colleagues have shown that the HA-tagged extracellular portion of Notch1 (NEC) dissociates from its intracellular counterpart following a 0.5mM EDTA treatment (129). The dissociated protein fragment can then be detected using an anti-HA antibody. This methodology cannot accurately characterize the surface expression of Notch1 molecules because unlike the intracellular protein samples where β-actin or GAPDH are frequently used as loading controls, the controls for these shed proteins are currently unavailable. Another previously utilized method is to nonspecifically label cell surface proteins with biotin, followed by purifying the biotinylated proteins using avidin-agarose. The avidin-bound biotinylated samples can then be analyzed on a protein gel. Rampal and colleagues used this cell-surface biotinylation experiment to measure the surface expression of Notch1 in COS-7 cells *in vitro* (125). This technique is often laborious and time consuming. More importantly, incomplete biotinylation of targeted proteins often occurs, which undermines the quantity of the cell surface protein. Together, the development of a sensitive and simple approach to detect Notch1 expression *in vivo* is justified.

To achieve this goal, we have devised a strategy to develop an animal model where Notch1 is tagged with a 3xFlag epitope at its amino terminus (3xFN). A series of *in vitro* experiments showed that epitope-tagged Notch1 functions equivalently to its wild-type counterpart and thus confirms the notion that 3xFlag epitope is suitable for my purpose (Figure 4-2 and Figure 5-1). To generate this animal model, targeted ESC clones in which a nucleotide encoding the 3xFlag epitope is installed between the signal peptide and the amino terminus of Notch1 have been produced (Figure 5-2) and injected in blastocysts. This strategy was used, instead of transgene, to maintain the genetic makeup of the Notch1 molecules. Following the implantation of ESCs carrying a flag-tagged Notch1 allele, we received 46 chimeras from two independent ESC clones, of which 17 were greater than 90% chimeric in coat color. Breeding of heterozygotes produced offspring whose genotypic ratio of wild-type/heterozygote/homozygote roughly followed the 1:2:1 Mendelian distribution. There were no observed phenotypic and behavior abnormalities in the progeny. Using animals carrying homozygous 3xFlag tagged Notch1 allele, we were able to characterize the surface expression of Notch1 in α-CD3 and α-CD28-stimulated naïve T cells using monoclonal α-Flag antibody. We can now use this mouse strain to examine the surface expression of Notch1 in T cell subsets. This approach will provide insight into the role of Notch1 in lineage commitment within T cell subsets.

Methods

Cell-based soluble Dll4 binding assay

HEK 293T cell lines were maintained in DMEM containing 10% FBS. Cells were transiently transfected with either pIRES-EYFP vector or pIRES-EYFP/WT-Notch1 or

pIRES-EYFP/3xFlag-Notch1 using Lipofectamine according to manufacturer's specifications. Briefly, 24 hours prior to the experiment, 1×10^6 cells were trypsinized into single cell suspension and replated in medium containing 10% FBS in the absence of antibiotics on a six-well plate. Six hours following the transfection, transfecting reagent was replaced DMEM supplemented with 1% FBS, in which cells grew for another 24 hours. On the day of experiment, transfected cells were harvested and washed with FACS staining solution (Hank's Balanced Salt Solution supplemented with 0.5% of albumin, 0.01% NaN_3 and 0.01 g/L of $CaCl_2$). After blocking with the Fc block (eBioscience) for 10 minutes on ice, cells were then evenly split into four aliquotes and incubated in 100 µl serum free medium containing either biotinylated anti-Flag isotype, biotinylated anti-Flag antibody (M2, Sigma Aldrich), human IgG or soluble Dll4 ligand (see previous chapters for preparation), for 30 minutes at room temperature. After washing with FACS staining solution, cells were incubated with either PE-conjugated streptavidin for anti-Flag antibodies or PE-conjugated anti-human IgG (γ-chain) antibody (Sigma Aldrich) for soluble Notch ligand for another 30 minutes on ice. Following two additional washes, these cells were analyzed using a FACS Aria (PerkinElmer). Normalized binding affinity of soluble Dll4 was reported as a ratio of mean fluorescence intensity for the soluble Notch ligand staining to that for EYFP expression.

Generation of 3xFlag-tagged Notch1 embryonic stem cell clones

Mice maintaining a targeted knock-in of flag-epitope in the Notch1 locus was generated according to the schematic diagram (Figure 5-2). The genomic DNA fragment containing exon1 and exon2 of Notch1 was first cloned from bacterial artificial chromosome (clone bMQ-333B24, Wellcome Trust Sanger Institute, Cambridge, UK)

into pBluescript. A 2.5kb DNA fragment containing floxed Neomycin cassette was then cloned into intron1 blunt-ended. Construct containing anti-sense floxed Neomycin insert was verified and selected. A two-stage polymerase chain reaction (PCR) strategy using QuikChange Site-Directed Mutagenesis was then utilized to introduce DNA encoding 3xFlag epitope into exon2, at which the junction between signal peptide and the amino-terminus of Notch1 is located (157). Briefly, forward (F) and reverse (R) primers containing sequences encoding 3xFlag epitope and flanking both 3' and 5' of Notch genomic sequence at the site of insertion were obtained:

F: 5'-
GACCTTCCCCCAGGCTTGAGAGACTACAAAGACCATGACGGTGATTATAAAG
ATCATGATATCGATTACAAGGATGACGATGACAAGTGCTCCCAGCCAAGTGG
GACC-3';

R: 5'-
GGTCCCACTTGGCTGGGAGCACTTGTCATCGTCATCCTTGTAATCGATATCAT
GATCTTTATAATCACCGTCATGGTCTTTGTAGTCTCTCAAGCCTGGGGGAAGG
TC-3'

Two separate extension reactions were then performed on Notch1 construct using each primer. Products from individual extension reaction were then combined for the standard site-directed mutagenesis expansion according to the manufacturer's specification. The final product was then sequenced to verify the presence of each exon and the insertion junction.

This targeting construct was then linearized and electroporated into R1 murine embryonic stem cells (ES). Transfected cells were co-cultured with irradiated embryonic fibroblasts in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS, 1000 units/ml of LIF and 300 μg/ml of G418. Individual G418-resistant ES clone was selected whose genotype was identified by Southern blotting using two separate DNA probes

located external to targeting construct (Figure 5-2a and b): 5’ probe (PCR-amplified with forward primer: 5’-TCCAAGCGTTGTAAGCAGTG-3’; reverse primer: 5’-ACCAGCCAACCAACAGAAAC-3’) for BamHI-digested genomic DNA (WT allele: 4.8 kb and knock-in allele: 3.6 kb) and 3’ probe (PCR-amplified with forward primer: 5’-TCTTCTGCTCAGCACTTGGA-3’; reverse primer: 5’-AGCAAGTTTTCTGCCAAGGA-3’) for HindIII-digested genomic DNA (WT allele: 9 kb and knock-in allele: 3 kb).

To avoid undesired inhibition of Notch1 expression by floxed-Neomycin cassette (158), two correctly targeted ES clones were transiently electroporated with pMC-Cre plasmid. Four days following the transfection reaction, individual clones were picked whose genotypes were then evaluated using PCR amplifications of genomic DNA template (Figure 5-3, floxed-Neomycin allele fragment with a size of 400 bp:
forward primer: 5’-CTTATTTCCCTTCCGGCTTC-3’;
reverse primer: 5’-TCTCGGGAAGGGGCTATTAT-3’).

Generation of mice maintaining homozygous targeted 3xFlag knock-in in theNotch1 locus

Upon verifying their genotypes, three ES clones (two of which containing Neomycin cassette and one without) were submitted to the University of Michigan Transgenic Animal Model Core for blastocyst microinjection. A total of 191 C57B/6NCrl blastocysts were injected. Upon receiving chimeras, we bred chimeric male whose coat color is greater than 90% chimeric to wild-type C57B/6J females. The 3xFlag-containing allele was confirmed in F1 progeny by Southern blotting of EcoRV-digested genomic DNA using the same PCR-amplified 3’ probe (see above) located external to the 3’ end

of the targeting construct (Figure 5-3, WT allele: >15kb, too large for clear detection and 3xF-knock-in allele: 2.5 kb). Genetically verified heterozygous F1 siblings were then bred to each other to generate homozygotes. WT, heterozygous and homozygous offspring were identified using PCR of the genomic DNA template (Figure 5-3,

forward primer: 5'-GGAGGCGATTAATGCTGTGT-3';

reverse primer: 5'-ACCCCATGCACAACTCTACC-3';

WT allele with a size of 250 bp and 3xFlag-knock-in allele: 320 bp).

Characterization of Notch1 surface expression in stimulated naïve T cells

Cell suspensions of spleens were prepared from adult WT and 3xFlag-Notch1 mice. Naïve $CD4^+$ T cell population was enriched from this preparation using the $CD4^+$ T Cells Isolation Kit according to the manufacturer's protocol (Miltenyi Biotec, Gladbach, Germany). Briefly, red blood cells were first removed using ammonium chloride containing buffer. Enucleated cells were stained with biotinylated antibody cocktail, followed by anti-biotin microbeads. Anti-body treated cell suspension was then applied onto the LS column. 2×10^6 cells from the flow through and subsequent washes were cultured onto the anti-CD3 coated six-well plate in the RPMI-1640 medium containing 10% of fetal bovine serum, 10 ng/ml of anti-CD28, 12.5 ng/ml of IL-2 and 10 units/ml of IL-12. Following the overnight culture, single cell suspension was harvested for the analysis of surface expressing Notch1.

Flow cytometric analysis

Expressions of cell surface markers were analyzed using FACS Aria (Becton Dickinson, Franklin Lakes, NJ). For the detection of surface markers, single cell suspensions were stained with fluorophore-conjugated antibodies in Hanks' Balanced

Salt Solution supplemented with 0.5% BSA for 20 minutes on ice. Appropriate isotype controls were included in each experiment. Data analysis was performed using FACSDiva software (Becton Dickinson).

Results

3xFlag-epitope on Notch1 does not interfere with soluble Dll4 binding in HEK 293T cells

The ability to characterize the cell surface expression of Notch1 receptor is critical to properly evaluate its function in T lymphopoiesis. Currently there are no antibodies to specifically recognize Notch1 on cell surfaces. In an effort to determine the stages of T lymphopoiesis where cell surface Notch1 is available, we would like to develop a mouse model whose amino terminus of Notch1 receptor is tagged by a 3xFlag epitope. This approach allows us to simply detect Notch1 surface expression using the monoclonal anti-Flag antibody and flow cytometry. Prior to generating this animal model, it is essential to examine whether the installed epitope changes the normal function of Notch1 receptor *in vitro*. In the previous chapter, we demonstrated that soluble Dll4 recognizes the surface expressing Notch1 in the presence of 3xFlag epitope. This was a quality measure instead of a quantity one since the binding was not normalized to the expression level of Notch1. Here we analyzed HEK 293T cells that were transiently transfected with either empty bicistronic EYFP expressing vector or a vector containing either wild-type Notch1 or 3xFlag-tagged Notch1, where EYFP functions as a surrogate marker for the transfection efficiency. Staining these transfected cells with the monoclonal anti-Flag antibody, we again demonstrated the specific

recognition of 3xFlag epitope on Notch1 by anti-Flag antibody (Figure 4-2b and 5-3). This data confirmed that Notch1 bearing a flag tag can be uniquely identified by the anti-Flag antibody. We then examined whether the addition of 3xFlag epitope at the amino terminus of Notch1 modifies the Notch and soluble Dll4 interaction. The binding was normalized to the transfection efficiency (EYFP expression level) to account for the differences in surface Notch1 available in each transfected HEK cell population. Cells transfected with either bicistronic expressing EYFP/WT-Notch1 or EYFP/3xFlag-Notch1 were positively stained by soluble Dll4 when compared to the negative control (Figure 5-3). Moreover, the binding of soluble Dll4 to WT-Notch1-expressing HEK cells was similar to that found in 3xFlag-tagged Notch1-expressing cells. Considered together with our previous finding that the installed exogenous peptide does not interfere with ligand-mediated Notch activity (Figure 4-2c), these data suggest that the 3xFlag epitope at the N-terminus of Notch1 can be used as a unique identifier for the surface expressing Notch1 protein.

Homozygosity for targeted insertion of 3xFlag epitope in the Notch1 allele produces no detectable abnormal phenotypic traits

Embryonic stem cell (ESC) clones containing homologous recombined insertion of oligonucleotides encoding the 3xFlag epitope were verified by sothern blotting (Figure 5-2). In total, we harvested 701 individual G418-resistant ESC clones, of which four were positively identified to have correct insertion of DNA fragment. Two of the four positive clones were then transiently transfected with Cre recombinase expressing plasmid to remove the floxed-Neomycin cassette. ESC clones with the floxed-Neomycin cassette

deleted were identified by polymerase chain reaction amplification (Figure 5-2). ESC clones containing positively targeted insertion with and without floxed-Neomycin cassette were both submitted for microinjections. Chimeric males derived from targeted ESC clones without the floxed-Neomycin cassette gave progeny that are heterozygous for the 3xFlag-Notch1 allele whereas those came from clones with the intact selection cassette did not give rise to heterozygous progeny. The offspring produced by mating two heterozygous mice roughly followed the Mendelian distribution. Of 59 progeny genotyped at weaning, 13 of them were wild-type, 32 of them were heterozygous and 14 of them were homozygous. Intercross of homozygous siblings derived from this mouse line produced the number of offspring per litter similar to those found in wild-type (between six and 10). There were no observed phenotypic and behavior abnormalities in the progeny. Together, adult mice maintaining targeted homozygous 3xFlag knock-in allele are similar to the wild-type.

Monoclonal anti-Flag antibody detects the surface expressing 3xFlag-tagged Notch1 in naïve T cells stimulated by anti-CD3 and anti-CD28 *in vitro*

Activated naïve T cells up-regulate the surface expression of Notch1 receptors (147). To examine whether surface expressing Notch1 in a primary cell culture can be detected using the monoclonal anti-Flag antibody, we isolated naïve $CD4^+$ splenocytes from mice containing targeted insertion of 3xFlag-tagged Notch1 allele. The purity of isolated cells was assessed by examining the surface expression of CD4 marker (Figure 5-4a). These cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and IL-12 in the culture prior to staining. To inspect the specificity of

the monoclonal anti-Flag antibody staining, naïve $CD4^+$ T cells purified from the spleens of wild-type mice were used as the negative control. When compared to the biotinylated isotype control, staining activated $CD4^+$ T cells bearing 3xFlag-tagged Notch1 receptors with the monoclonal anti-Flag antibody were enhanced by approximately 4.8-fold (mean fluorescence intensity (MFI) ratio: 901/185) (Figure 5-4b). The staining of anti-Flag antibody was not observed in those isolated from the wild-type mice (MFI = 204). These results indicate the flag epitope specificity of monoclonal anti-Flag antibody in a primary cell culture.

Discussion

In this study, we developed a simple and specific assay to detect the surface expression of Notch1 using a readily available monoclonal anti-Flag antibody. Our data show that the installation of the 3xFlag epitope does not grossly interfere with the normal function of Notch1 receptor, including its transportation to the cell surface, interaction with ligands and the down-stream activity. Moreover, the growth and development of mice maintaining the targeted insertion of the flag epitope in the Notch1 locus are not disrupted. Together, this mouse model provides a unique opportunity for the study of the cell surface expression of Notch1 receptors.

The surface expression pattern of Notch1 in thymocyte development is of considerable interest. Notch1 is indispensable for the T lineage commitment of the thymic progenitors at the early stage. However, whether Notch1 plays role in each subsequent lineage specification, such as αβ versus γδ, CD4 versus CD8 and Th1 versus Th2, is still unclear. The presence of Notch1 in thymocytes at different developmental

stages has been documented extensively using RT-PCR. These studies showed that Notch1 mRNA progressively increases in quantity from the earliest intra-thymic progenitors to the double-negative 3 ($CD4^-CD8^-CD25^+CD44^-$) stage followed by a markedly reduction and remains throughout the rest of the T cell developmental process (156). How well the differential Notch1 mRNA expression level at different T mature stages correlates with the surface expressing Notch1 protein remains to be determined. Armed with our mouse model whose Notch1 is tagged with 3xFlag epitope on its extracellular domain, studies to elucidate the availability of surface Notch1 at the different developmental stages are now achievable using the anti-Flag antibody.

Upon engaging with APCs, naïve helper T cells undergo differentiation and proliferation. Activation of these cells is controlled by numerous molecular events. The interaction between T cell receptor and major histocompatibility complex class II molecules on APC initiates a $CD4^+$ T cell response. The stimulation through T cell receptor alone is inadequate to activate naïve $CD4^+$ T cells without the support of co-stimulatory signals, however. This is achieved through the CD28 surface molecule. In addition to T cell receptors and CD28 proteins, other surface molecules including lymphocyte function-associated antigen-1, CD2, OX40 and inducible co-stimulator receptors have all been demonstrated to regulate this process (159-162). Activation of these surface molecules leads to a diverse intracellular events, which consequently promote the activity of transcription factors to drive the expression of necessary transcripts. Recent reports indicated that Notch1 activity may play a role in T cell activation, proliferation and cytokine production (147, 148). By characterizing the surface expression of Notch1 on activated $CD4^+$ splenocytes in the primary culture, we

have revealed the enhanced availability of Notch1 for the participation of naïve T cell activation. Our finding should be followed by in-depth analysis of Notch1 activity in both differentiation and proliferation of activated peripheral T cells. Moreover, studies on whether and how Notch1 regulates the activation of these T cells during bacterial infection *in vivo* are needed.

Notes to Chapter V

I want to thank Bronislawa Petryniak for performing the *in vitro* naïve T cell stimulation assay and the flag staining experiment shown in Figure 5-4. I also want to thank Jay Myers for providing training and expertise in generating a genetically engineered mouse model. Moreover, I want to express my appreciation to the University of Michigan Transgenic Animal Model Core for the mouse chimera production.

Figure 5-1

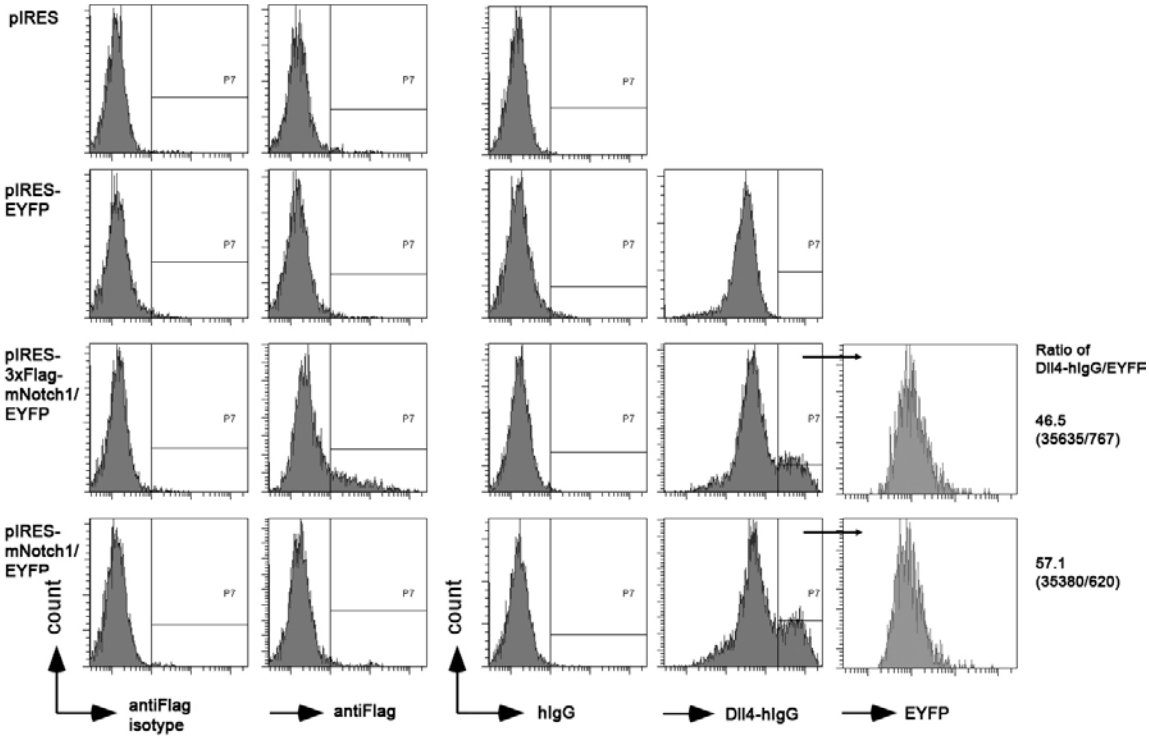
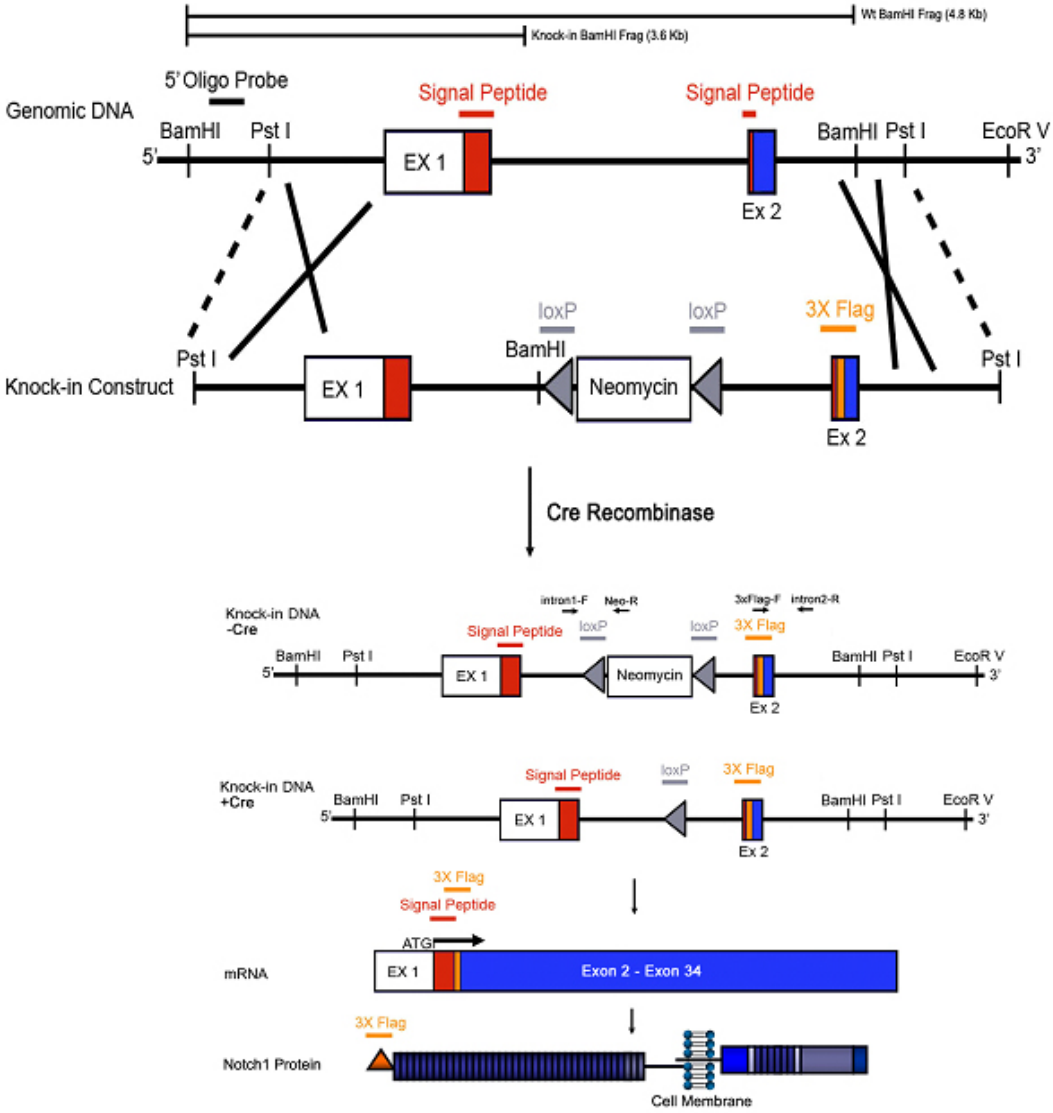


Figure 5-1. Quantifying the binding of soluble Dll4 to WT and 3xFlag–tagged mNotch1. Staining of HEK 293T cells transiently transfected with empty vector (pIRES) or bicistronic EYFP expressing construct with nothing or 3xFLAG-tagged mNotch1 or wild-type mNotch1 with monoclonal anti-FLAG antibody, isotype-matched negative control antibody, Dll4-IgG fusion protein, or a human IgG negative control (see Materials and Methods). Binding affinity of soluble Dll4 is reported as a ratio of the MFI of Dll4 staining over that of EYFP expression. Data are representative of three independent experiments. MFI: mean fluorescence intensity.

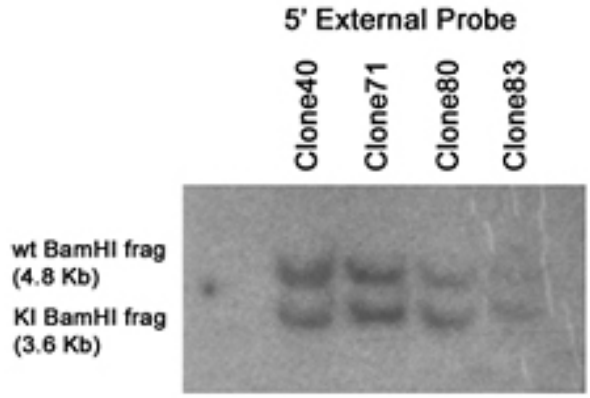
Figure 5-2. Generation of embryonic stem cell clones containing correctly targeted insertion of 3xFlag epitope in the Notch1 locus. a. Schematic diagram illustrating the structure of WT and 3xFlag-inserted Notch1 locus with and without the floxed Neomycin cassette in the intron 1. The nucleotides encoding the 3xFlag epitope was inserted in the exon 2, at the junction between signal peptide and the amino-terminus of Notch1. **b.** Southern blot analysis of embryonic stem cell (ESC) clones containing correctly targeted 3xFlag-tagged insertion (previously identified). Genomic DNA from each clone was digested with BamHI restriction endonuclease and probed using a DNA probe external to the 5' end of the knock-in construct. WT and KI (knock-in) BamHI fragments are 4.8 Kb and 3.6 Kb in size, respectively. **c.** Polymerase chain reaction analysis of ESC clones transiently transfected with Cre recombinase expressing construct. The deletion of floxed neomycin cassette fragments for each ESC clone was verified by PCR analysis using the neomycin specific primer set. 3xFlag epitope for each corresponding ESC cloning was also checked by PCR amplification using the exon2 flanking primer set. C: control PCR reaction.

Figure 5-2

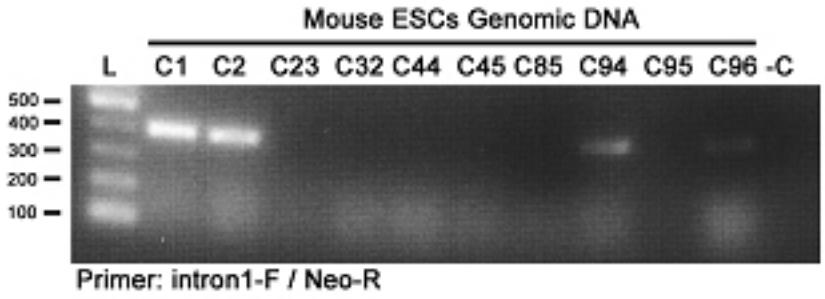
a.

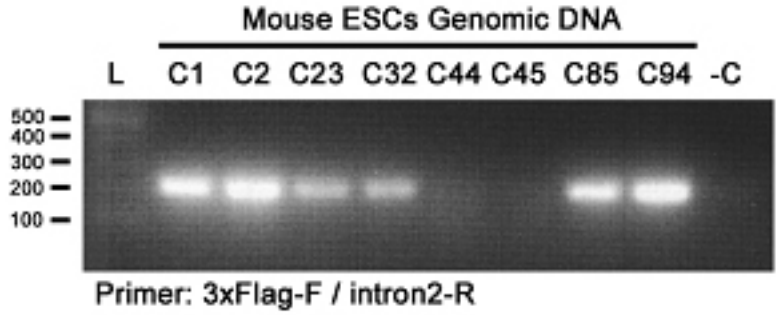

Wt BamHI Frag (4.8 Kb)
Knock-in BamHI Frag (3.6 Kb)
5' Oligo Probe
Genomic DNA
BamHI
Pst I
Signal Peptide
Signal Peptide
BamHI Pst I
EcoR V
5'
3'
EX 1
Ex 2
loxP
loxP
3X Flag
BamHI
Pst I
Pst I
Knock-in Construct
EX 1
Neomycin
Ex 2
Cre Recombinase
Knock-in DNA
-Cre
intron1-F
Neo-R
3xFlag-F
intron2-R
Signal Peptide
loxP
loxP
3X Flag
BamHI
Pst I
5'
EX 1
Neomycin
BamHI Pst I
EcoR V
3'
Ex 2
Knock-in DNA
+Cre
Signal Peptide
loxP
3X Flag
BamHI
Pst I
5'
EX 1
BamHI Pst I
EcoR V
3'
Ex 2
3X Flag
Signal Peptide
ATG
mRNA
EX 1
Exon 2 - Exon 34
3X Flag
Notch1 Protein
Cell Membrane

b.


5' External Probe
Clone40
Clone71
Clone80
Clone83
wt BamHI frag
(4.8 Kb)
KI BamHI frag
(3.6 Kb)

c.


Mouse ESCs Genomic DNA
L
C1
C2
C23
C32
C44
C45
C85
C94
C95
C96
-C
500
400
300
200
100
Primer: intron1-F / Neo-R


Mouse ESCs Genomic DNA
L
C1
C2
C23
C32
C44
C45
C85
C94
-C
500
400
300
200
100
Primer: 3xFlag-F / intron2-R

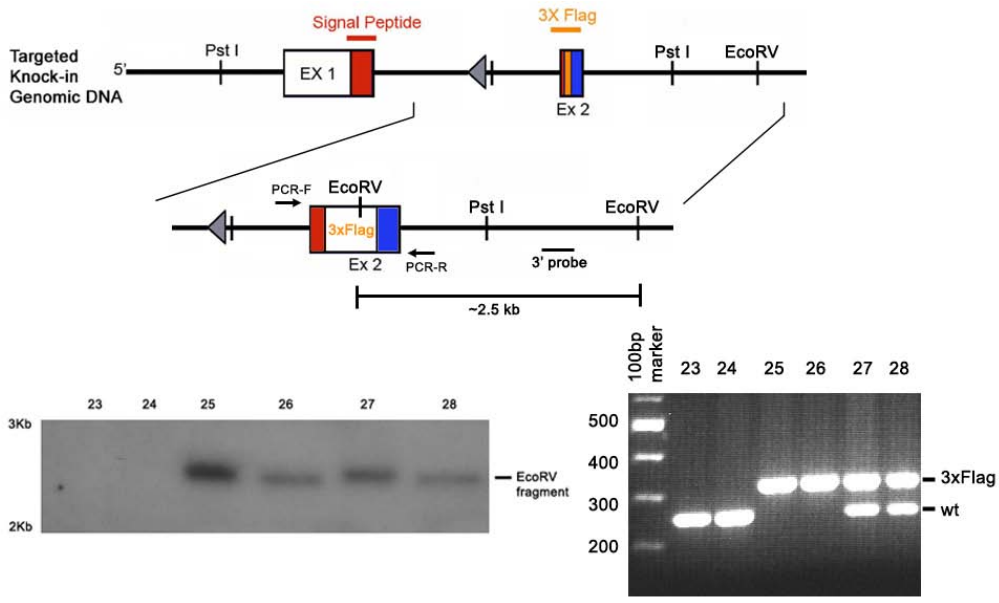
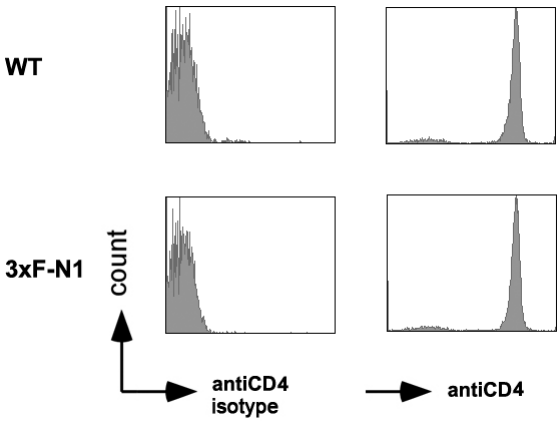


Figure 5-3. Genotyping of mice maintaining targeted insertion of 3xFlag epitope in the Notch1 locus. Diagram demonstrating the genomic DNA structure of 3xFlag containing Notch1. Southern blot analysis of EcoRV-digested genomic DNA isolated from the progeny of the mating between two heterozygous parents using a DNA probe located external to the 3' end of the knock-in DNA construct. The 2.5 Kb fragment was indicative the presence of the 3xFlag allele. Polymerase chain reaction analysis of the same progeny using primers specific for the amplification of the entire exon 2. 3xFlag allele yielded a 320 bp fragment while WT allele gave rise to a fragment with a size of 250 bp.

a.



b.

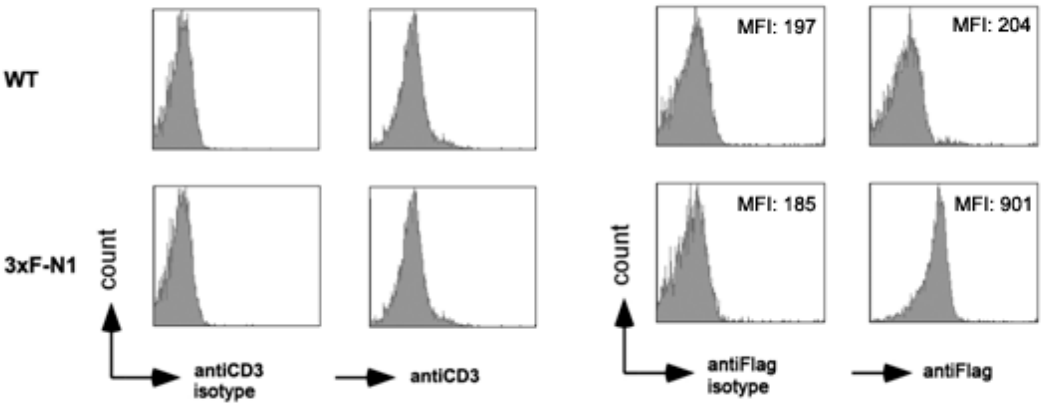


Figure 5-4. Detecting Notch1 in activated naïve CD4$^+$ T cells. a. Staining of purified CD4$^+$ cells isolated from WT and 3xF-N1 mice. **b.** Staining activated naïve CD4$^+$ T cells with anti-CD3 and monoclonal anti-Flag antibodies. WT – wild-type; 3xF-N1 – mouse carrying homozygous 3xFlag-tagged Notch1 allele. MFI: mean fluorescence intensity

CHAPTER VI

Conclusions

Since inception, the foundation of modern biology has stemmed from the concept of the sequential flow of biologic information from DNA to mRNA to protein. This information transfer system is dependent on the cellular capacity to precisely decode DNA templates. The ability to manipulate DNA molecules allows scientists to gain tremendous insight into the biology of nucleic acids and proteins. This, however, generates a propensity to understand the biological systems in terms of these two classes of molecules. Considered together with the lack of tools in elucidating the biological roles of sugar molecules, the study of carbohydrates has lagged behind.

As mentioned previously, carbohydrates function as a repository containing an enormous amount of biological information. Their roles are multifaceted. As freestanding entities, individual sugar molecules or their aggregates can serve in energy storage, structural support and cellular recognition. When these sugar molecules are fastened to proteins or lipids, the scope of their roles expand to cellular interaction, signaling amplification, protein structural integrity and regulatory switch. There are times, however, when sugar molecules are dispensable in biological activities. Given that the presence of these molecules enriches the biological complexities of living organisms, there are innumerable opportunities to uncover the various roles of sugar molecules.

Fucose molecules are abundantly present in a wide variety of organisms. Evidence from studies in mammals has demonstrated that fucose is crucial in leukocyte trafficking, red blood cell phenotyping, host-microbe interaction and signaling event regulation. The biosynthesis of fucose was first elucidated in the early 60's (163) and

since then, the biology of fucose molecules has slowly been unearthed. Initially the role of fucose was seen in the setting of its role in the terminal modification of glycans. It was not until recently that the direct linkage between fucose and protein peptide was established. These fucose-containing glycoconjugates are found on Notch receptors, Notch ligands, Cripto-1, tissue plasminogen factor, clotting factors and several other surface expressing and secreted molecules. To elucidate the role of fucose-dependent Notch biology, we studied a mouse maintaining a targeted deficiency in the synthesis of GDP-fucose, the universal substrate for all fucosyltransferases. Our data suggest that Notch activity is critically dependent on the presence of fucose-containing glycans. Our observations further implicate fucose-dependent Notch activity in the hematologic lineage specification including myeloid, T-lymphoid and splenic B-lymphoid subset lineages. While these results are derived from a series of well-controlled experiments, our findings do not rule out the possible contributions of other fucose-containing glycoproteins. This is partly due to limitations in our experimental mouse model that carries a global cellular fucosylation defect, which makes it difficult to ascribe a concrete function to the locus. To uncover the biological role of *O*-fucose glycans in Notch activity and mature blood cell development with an increased degree of specificity, we focus our attention on the function of Pofut1.

Using a murine embryonic stem cell line and an animal model maintaining a targeted deficiency in the Pofut1 locus, our findings illustrate that Pofut1 activity promotes T-cell and splenic B-cell subset determination while suppressing myeloid-lineage specification. Reversal of the aberrant hematologic phenotypes in Pofut1-deficient animals by reconstituting transplanted bone marrow progenitors with

constitutively active Notch1 molecules assign a role for *O*-fucose dependent Notch activity in the formation of mature blood cells. Given that previous studies investigating the effect of Notch activity in blood cell lineage differentiation demonstrated that Notch1 is indispensable for the development of mature T lymphocytes whereas Notch2 mediates differentiation of the marginal zone B cell subset in the spleen, our finding strongly supports the significance of *O*-fucose-dependent Notch activation in lymphoid lineage specification.

Although tremendous strides have been made in characterizing the functional role of *O*-fucose in the context of hematopoiesis, many critical questions still remain unanswered. I would like to take this opportunity to discuss these issues in details in the remaining section:

Notch in hematopoietic stem cell fate

Our data examining the role of Notch in hematopoietic stem cell (HSC) self-renewal is not presented previously because it is outside the scope of my dissertation. However, this topic deserves special attention as the expansion of HSC's is critical for the success of its therapeutic applications. HSCs are a rare cell population. These progenitors are not only required to differentiate into all lineages of mature blood cells, but also to produce additional HSCs to maintain the stem cell pool. The process of self-renewal is intricately regulated by the marrow microenvironment via various secreted and surface-bound ligand-receptor signaling pathways, including Wnt/β-catenin, prostaglandin E2/EP4 G-protein coupled receptor, hedgehog, bone morphogenetic protein and possibly Notch (63). To address the functional role of the Notch pathway in HSC homeostasis, we examined the HSC population in mice deficient in cellular fucosylation.

Our data indicate that inhibition of *O*-fucosylation of Notch produces a decreased HSC distribution defined as $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSKs) (110). This decrease is largely attributed to the reduction of multipotent progenitors, or LSKs expressing Flt3 receptors. Moreover, individual HSCs that are functionally *O*-fucose-deficient are less capable of forming multi-lineage colonies when compared to the wild-type progenitors. Consistent with our observations, others have reached similar conclusions using different animal models. In mice, Notch activity is enhanced and down-regulated in HSCs undergoing proliferation and differentiation, respectively (164). HSCs expressing either constitutively active Notch1 or Notch-specific transcript Hes1 exhibit augmented self-renewal capability (77, 165). Mice with enhanced Notch activity, through the support of osteoblasts bearing J1 ligand, yield an expansion of the HSC compartment (166). In contrast, targeted ablation of Jagged1 does not appear to alter HSCs homeostasis (81). Moreover, blockade of all Notch activity in HSCs using either dominant-negative MAML molecules or targeted deficiency at the CSL locus does not result in a retardation of their self-renewal ability (109). Although the discrepancy remains to be sorted out, it is conceivable that other Notch receptors and ligands compensate for the loss of Jagged1 ligand in the bone marrow. In the latter case, it is possible that Notch maintains the HSC population in a CSL-independent fashion given that cross-talk between Notch and other signaling pathways governing HSC self-renewal, such as Wnt, Sonic hedgehog (Shh) and Smad, has been documented extensively (63). To clearly establish the role of Notch signaling in adult HSC maintenance under physiological conditions, additional work to address the function of bone marrow progenitors isolated from a mouse model maintaining conditional deletions of all four Notch receptors is necessary.

In addition to maintaining the HSC population, it is possible that Notch functions as a regulatory switch that supports the entry of HSCs into lineage restricted progenitors. Our data suggest that Notch maintains the balance between lymphoid and myeloid progenitor synthesis in an *O*-fucose dependent fashion. Absence of *O*-fucose moieties on Notch results in an expansion of myeloid lineage-committed progenitors and a decreased common lymphoid progenitor compartment in the bone marrow. Similarly, individual progenitors isolated from mice carrying the cellular fucosylation defect give rise to an increased frequency of myeloid colonies but a decreased number of lymphoid colonies *in vitro*. This reduced ability to differentiate into the lymphoid progenitor population further contributes to the faulty T cell development observed in Pofut1-deficient mice. The notion that *O*-fucose dependent Notch activity regulates the lymphoid versus myeloid lineage specification in HSCs deserves further study. A recent study demonstrates that ex vivo chemical modification of fucose-containing glycoconjugates on surface expressing the CD44 glycoprotein enhances the homing potential of mesenchymal stem cells (168). Using a similar approach, we can possibly alter the lineage differentiation potential of bone marrow progenitors by enzymatically modifying the *O*-fucose structure on Notch *ex vivo*. This will further the therapeutic relevance of bone marrow progenitors as the demand for these cells in clinical applications has increased tremendously. Moreover, the underlying mechanisms by which Notch regulates lineage determination in marrow progenitors requires additional assessment in order to provide further insight into the interaction between progenitors and the bone marrow microenvironment.

Notch in the development of T lymphocytes

T cell maturation occurs in the thymus. Early thymic progenitors originating in the bone marrow travel to the cortico-medullary junction of the thymus where the interaction between progenitors and stromal cells (via intercellular adhesion molecules) permits entry. As shown in the previous chapters, Notch1 and lunatic fringe glycosyltransferase (Lfng) may facilitate the entry of thymic progenitors into the thymus. Biochemically, Lfng modification of *O*-fucose moieties on Notch1 leads to the enhanced binding affinity between Notch1 and Delta-like ligands. Moreover, Pofut1-deficient donor progenitors do not contribute to the mature T cell population in the wild-type recipients only if they are competing against wild-type donor cells. Considering our findings together with the presence of Lfng in the early thymic progenitors and the fact that Lfng-null progenitors generate few mature thymocytes (118), *O*-fucose moieties on Notch provides thymic progenitors a competitive advantage for access to Delta-like ligands whose expression is functionally limited in the thymic niche. This fucose-dependent interaction between Notch and Delta-like ligands in the thymic niche may be critical for T lineage commitment and survival. Following the initial Notch1-dependent T lineage determination, double negative thymocytes migrate toward the medulla for further Notch1-independent intrathymic development. What remains unclear is how thymic progentiors vacate the niche. Whether this is a passive regulation such that the internalization of Notch receptors following Notch activation disrupts the Notch-Delta-like ligand anchoring or activated Notch signaling up-regulates cell motility mechanism requires additional analysis. Moreover, Delta-like ligand-mediated Notch signaling has been connected to peripheral T cell activation. Thus, it is critical to determine the

physiological relevance of *O*-fucose molecules and Lfng modifications in the peripheral immune response.

Upon entering the thymus, progenitors intimately interact with the thymic stroma, where Notch ligands, Dll1, Dll4 and Jagged2 are expressed throughout. This interaction has proven to be critical in guiding the progenitors through a defined path to reach their mature stage. The extracellular domain of Notch ligand contains multiple tandem EGF-like repeats. Missense mutations in these motifs are associated with an autosomal dominant developmental disorder in humans (121, 169). Similar to their receptor counterparts, each of the EGF-like domains can be modified by *O*-fucose molecules and further targeted for the Fringe elongations. These *O*-fucose moieties are likely to facilitate the proper binding to Notch receptors. It is possible that these fucose-containing glycans are involved in functions reminiscent of those on Notch receptors including the interaction between Notch receptor and ligand as well as the surface expression of the ligands, both of which contribute to the development of T cells. To this end, data from our bone marrow transplant experiments show that the defective thymocyte development phenotype is elicited only if both thymic progenitors and the thymic stromal cells bear targeted deficiency at the Pofut1 locus. This observation suggests that proper Notch activity driving thymic progenitors to T lineage is equally contributed by Notch and its thymic ligands, Dll4 and possibly Dll1 and Jagged2, in an *O*-fucose dependent manner. Our observation assigns a functional property to *O*-fucose glycans on Notch ligand. Given that much is known about the biosynthesis of *O*-fucose containing glycoconjugates on Notch ligands, the future challenge is to identify and characterize the role of these glycans in both Notch ligand expression and Notch signaling. To address the functional

role of *O*-fucosylation sites on Notch ligands, dynamics of thymocyte and stromal cell interactions in the thymus isolated from Pofut1-deficient mice will be measured *ex vivo* using two-photon microscopy, a technique that was recently developed by Robey and colleagues (170).

Notch in the development of myeloid lineage cells

The maturation of myeloid lineage cells occurs in the bone marrow. This process is regulated by both growth factors and the bone marrow microenvironment. Although several molecules promoting the proliferation and differentiation of neutrophils have been identified, the key signaling pathway that instructs myeloid lineage commitment remains elusive. As stated previously, the Notch pathway is necessary in T lineage commitment. However, studies both supporting and undermining the notion that Notch inhibits myelopoiesis make its role in this lineage commitment unclear. Previous studies suggesting that Notch is not involved in myelopoiesis were derived from inactivation of Notch1 or Notch2 or a combination of Notch1 and Jagged1 (44, 81). In contrast, our observations in mice harboring either a global fucosylation or *O*-fucosylation deficiency suggest that *O*-fucose dependent Notch activity is critical for suppressing overt development of mature myeloid cells. Given that Notch1, Notch2 and Notch3 are expressed in the bone marrow progenitors, the discrepancy between our finding and previous reports may be due to the redundant functions of Notch homologs. To confirm the biological role of Notch activity in myelopoiesis, functional correlates of compound Notch homologs deficiencies on the modulation of lymphoid versus myeloid lineage diversification are needed.

Despite our data showing the negative impact of *O*-fucose dependent Notch activity on myelopoiesis, the underlying cellular mechanisms remain unclear. One possibility is that Notch activity favors the development of common lymphoid progenitors (CLP) at the expense of common myeloid progenitors (CMP) from multi-potent progenitors. Indeed, Pofut1-deficient mice express a noticeable increase in the CMP population with a relatively reduced CLP compartment in the marrow. As our data suggest, this enhanced CMP population is likely due to the increased expression of myeloid specific transcripts, including Gfi-1, C/EBPα and PU.1 with reduced Notch targets. In the absence of Notch activity, to uncover how biological information is precisely transferred through these transcripts, thereby promoting CMP specification at the expense of CLP, it is critical to identify cellular mechanisms by which Notch activity down-regulates the expression of myeloid specific transcription factors. Additional studies are also needed to clarify the molecular functions of Gfi-1, C/EBPα and PU.1 in promoting myeloid differentiation. Although our data show that inhibition of the Notch pathway facilitates the myeloid determination, we cannot exclude the possibility that CMP accelerates and proliferates towards the terminal myeloid lineage in the absence of Notch activity. Thus, to elucidate the ability of Notch to suppress myeloid differentiation potential, careful analyses of proliferative ability and colony formation aptitude towards different lineages from a single bone marrow progenitor with and without Notch function are necessary.

Notch1 in Regulating the Development of T-lymphocyte Subsets

One of the goals in my study is to elucidate the functional roles of Notch1 in the development of thymocytes. Although Notch1 is critical in T lineage commitment and the formation of a pre-TCR complex which permits further differentiation of T cells, its function in the subsequent lineage differentiation stages is still unclear. As previously mentioned, this ambiguity is partly due to the difficulty in characterizing the surface expression of Notch1 in T cell subsets. Currently there are no reagents available for detecting Notch1 on the cell surface using flow cytometry. In order to determine the stages of T lymphopoiesis where Notch1 is available to activate downstream signaling events, we generated an animal model (3xF-N1) that maintains a targeted insertion of an oligonucleotide encoding the 3xFlag epitope at the amino terminus of Notch1.

In the previous chapter, I presented data demonstrating our ability to characterize the surface expression of Notch1 in cells isolated from this mouse model using the monoclonal α-Flag antibody. As we continue to move forward with our study, we want to define cell-type specific expression patterns of Notch1 and to correlate these patterns to Notch activity in the immune system using flow cytometry. To address these goals, a mouse strain which simultaneously maintains a homozygous 3xF-N1 allele and a Notch-reporter transgene (3xF-N1 reporter) will be generated. This Notch-reporter transgene is composed of four tandem copies of C-promoter binding factor 1 (CBG1), followed by the SV40 minimal promoter and an enhanced green fluorescent protein (EGFP) sequence. The expression of EGFP in these animals functions as a surrogate marker for Notch activity. Previous studies have shown differential expression patterns of EGFP during T cell development in this reporter mouse model. Following the generation of the 3xF-N1 reporter mouse strain, the surface expression level of Notch1 in $CD4^{-}CD8^{-}$ (DN)

immature thymus subsets, including DN1 ($CD44^+$ $CD25^-$ $CD117^+$), DN2 ($CD44^+CD25^+CD117^+$), DN3 ($CD44^-CD25^+CD117^-$) and DN4 ($CD44^-CD25^-CD117^-$) and $CD4^+CD8^+$ (DP), $CD4^+CD8^-$, $CD4^-CD8^+$ mature thymus subsets in the adult mice will be quantified using the monoclonal anti-Flag antibody. The surface expression pattern of Notch1 will then be correlated with its mRNA expression level in these subsets. Previous studies demonstrated that the quantity of Notch1 mRNA gradually increases to the DN3 stage and reduces considerably thereafter. We will further characterize Notch activity in each thymus subset by the expression of enhanced green fluorescent protein. We will also analyze the expression levels of Notch1 mRNA and surface protein in thymi isolated from mouse embryos at the developmental age of 14-16 days. Similar to the analysis of the adult thymus, the expression levels of Notch1 will be categorized based on the immature and mature thymus subsets. This study will provide insight into the functional roles of Notch1 in lineage commitment within T cell subsets in both adult and neonatal animals.

Notch signaling has been implicated in the activation of peripheral T cells. Our previous observation is consistent with this notion that antibody stimulated naïve $CD4^+$ splenocytes up-regulate the surface expression of Notch1 receptors *in vitro*. To further elucidate the functional role of Notch1 in the periphery, we will start by identifying the expression pattern of Notch1 on naïve and activated $CD4^+$ and $CD8^+$ T cells after either an intravenous or intra-peritoneal bacterial or viral challenge in 3xF-N1 reporter mice. We will then adoptively transfer $CD4^+$ T cells isolated from inducible Notch1-deficient donors (available from another lab) that are previously immunized with a known bacterial peptide (such as *H. pylori* urease) into either SCID, Rag2-null or lethally irradiated

syngeneic recipients. The bacterial specific cellular immune response will then be analyzed in challenged recipients. Using Notch1-null derived donor cells, we will be able to assess the physiological relevance of Notch1 function in the peripheral T cell activation after TCR stimulation. To examine the functional relevance of Notch signaling in TCR-mediated T cell activation, we will further perform adoptive transfer of Pofut1-deficient $CD4^+$ T cells.

Aside from the conventional $CD4^+$ and $CD8^+$ T cell lineages, Notch1 has been functionally associated with other immune system cell types. For instance, functional NK and NKT cells can be derived from embryonic stem cells when they are co-cultured with bone marrow stromal cells expressing Notch ligands (171, 172). Moreover, Notch1, along with TGFbeta1, has been implicated in the maintenance of peripheral regulatory T cells (173). To elucidate the functional role of Notch1 in the less frequently occurring T cell relatives, a study aimed to characterize the expression pattern of Notch1 in NK cells, CD1d-restricted NKT cells, regulatory T cells and intestinal epithelial T cells using our 3xF-N1 animal model is warranted. Physiological significance of Notch1 in each of these unconventional T lineages, whether it is lineage commitment, cellular survival or functional activation, remains to be determined.

Final words

The evolutionary process has led *Drosophila* Notch to progress into four homologues and five ligands in mammals. The combinations of four receptors and five ligands with multiple post-translational modifications, including *O*-fucosylation and Fringe glycosylation, provide a more precise regulation of signals during the

developmental process. Although different receptor-ligand arrangements contribute to a greater complexity which has made it challenging to evaluate the role of the Notch pathway in biological events, studies aimed to uncover the mechanisms of *O*-glycan dependent Notch activity in the development of mature blood cells will provide new insight into hematopoiesis. Moreover, further understanding towards the regulations of Notch signaling may lead to new strategies of stem cell application and cancer treatment.

BIBLIOGRAPHY

1. Stanley, P. Schachter, H. and Taniguchi, N. (2008) N-Glycans. In A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart and M.E. Etzler (Eds.) Essentials of Glycobiology 2nd Edition (pp. 101-115). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

2. Granovsky, M., Bielfeldt, T., Peters, S., Paulsen, H., Meldal, M., Brockhausen, J. and Brochhausen, I. (1994) UDP-galactose:glycoprotein-N-acetyl-d-galactosamine 3-β-d-galactosaminyltransferase activity synthesizing O-glycan core 1 is controlled by the amino acid sequence and glycosylation of glycopeptide substrates. Eur J Biochem 221:1039–1046.

3. Kuhns, W., Rutz, V., Paulsen, H., Matta, K.L., Baker, M.A., Barner, M., Granovsky, M. and Brockhausen, I. (1993) Processing O-glycan core 1, Gal ß1–3GalNAc -R. Specificities of core 2, UDP-GlcNAc:Gal ß1–3 GalNAc-R(GlcNAc to GalNAc) ß6-N-acetylglucosaminyltransferase and CMP-sialic acid:Gal ß1–3GalNAc-R 3-sialyltransferase. Glycoconj J 10:381-394.

4. Vavasseur, F., Yang, J.M., Dole, K., Paulsen, H. and Brockhausen, I. (1995) Synthesis of O-glycan core 3: characterization of UDP-GlcNAc: GalNAc-R beta 3-N-acetyl-glucosaminyltransferase activity from colonic mucosal tissues and lack of the activity in human cancer cell lines. Glycobiology 5:351-357.

5. Yeh, J.C., Ong, E. and Fukada, M. Molecular cloning and expression of a novel β-1,6-N-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. J Biol Chem 1999; 274: 3215–3221.

6. Wopereis, S., Lefeber, D.J., Morava, E. and Wevers, R.A. (2006) Mechanisms in protein O-glycan biosynthesis and clinical and molecular aspects of protein O-glycan biosynthesis defects: a review. Clin Chem 52:574-600.

7. Herscovics, A. (1999) Importance of glycosidases in mammalian glycoprotein biosynthesis. Biochim Biophys Acta 1473:96-107.

8. Varki, A. (1996) "Unusual" modifications and variations of vertebrate oligosaccharides: are we missing the flowers for the trees? Glycobiology 6:707-710.

9. Funderburgh, J.L. (2002) Keratan sulfate biosynthesis. IUBMB Life 54:187-194.

10. Shi, W.X., Chammas, R. and Varki, A. (1996) Linkage-specific action of endogenous sialic acid O-acetyltransferase in Chinese hamster ovary cells. J Biol Chem 271:15130-15138.

11. Freeze, H.H. and Ichikawa, M. (1995) Identification of N-acetylglucosamine-(-1-phosphate transferase activity in Dictyostelium discoideum: an enzyme that initiates phosphoglycosylation. Biochem. Biophys Res Commun 208:384-389.

12. Molinari, M. (2007) N-glycan structure dictates extension of protein folding or onset of disposal. Nat Chem Biol 3:313-320.

13. Potter, B.A., Hughey, R.P. and Weisz, O.A. (2006) Role of N- and O-glycans in polarized biosynthetic sorting. Am J Physiol Cell Physiol 290:C1-C10.

14. Varki, A. and Lowe, J.B. (2008) Biological Roles of Glycans. In A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart and M.E. Etzler (Eds.) Essentials of Glycobiology 2nd Edition (pp. 75-88). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

15. Komfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. Annu Rev Cell Biol 5:483-525.

16. Xu, H., Manivannan, A., Jiang, H.R., Liversidge, J., Sharp, P.F., Forrester, J.V. and Crane, I.J. (2004) Recruitment of IFN-gamma-producing (Th1-like) cells into the inflamed retina in vivo is preferentially regulated by P-selectin glycoprotein ligand 1:P/E-selectin interactions. J Immunol 172:3215-3224.

17. Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M. and Sunshine, J. (1988) The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. Science 240:53–57.

18. Hu, H., Tomasiewicz, H., Magnuson, T. and Rutishauser, U. (1996) The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. Neuron 16:735–743.

19. Seki, T., Namba, T., Mochizuki, H. and Onodera, M. (2007) Clustering, migration, and neurite formation of neural precursor cells in the adult rat hippocampus. J Comp Neurol 502:275–290.

20. Camus, D. and Hadley, T.J. (1985) A Plasmodium falciparum antigen that binds to host erythrocytes and merozoites. Science 230:553-556.

21. Duraisingh, M.T., Maier, A.G., Triglia, T. and Cowman, A.F. (2003) Erythrocyte-binding antigen 175 mediates invasion in Plasmodium falciparum utilizing sialic acid-dependent and -independent pathways. Proc Natl Acad Sci USA 100:4796-801.

22. Jaeken, J. and Matthijs, G. (2007) Congenital disorders of glycosylation: A rapidly expanding disease family. Annu Rev Genomics Hum Genet 8: 261–278.

23. Ley, K. (2003) The role of selectins in inflammation and disease. Trends Mol Med 9: 263–268.

24. Lefer, D.J., Flynn, D.M., Phillips, M.L., Ratcliffe, M. and Buda, A.J. (1994) A novel sialyl LewisX analog attenuates neutrophil accumulation and myocardial necrosis after ischemia and reperfusion. Circulation 90:2390-2401

25. Dong, Z.M., Chapman, S.M., Brown, A.A., Frenette, P.S., Hynes, R.O. and Wagner, D.D. The combined role of P- and E-selectins in atherosclerosis. (1998) J Clin Invest 102: 145-152.

26. An, G., Wang, H., Tang, R., Yago, T., McDaniel, J.M., McGee, S., Huo, Y. and Xia, L. (2008) P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. (2008) Circulation 117:3227-3237.

27. Mulligan, M. S., Miyasaka, M., Tamatani, T., Jones, M.L., and Ward, P.A. (1994) Requirements for L-selectin in neutrophil-mediated lung injury in rats. J. Immunol. 152: 832-840.

28. Schürmann, G.M., Bishop, A.E., Facer, P., Vecchio, M., Lee, J.C., Rampton, D.S. and Polak, J.M. (1995) Increased expression of cell adhesion molecule P-selectin in active inflammatory bowel disease. Gut 36:411-418.

29. Kim, Y.J., Borsig, L., Varki, N.M. and Varki, A. (1998) P-selectin deficiency attenuates tumor growth and metastasis. Proc Natl Acad Sci U S A 95:9325-30.

30. Chen, S. and Fukuda, M. (2006) Cell type-specific roles of carbohydrates in tumor metastasis. Methods Enzymol 416:371–380.

31. Fuster, M.M. and Esko, J.D. (2005) The sweet and sour of cancer: Glycans as novel therapeutic targets. Nat Rev Cancer 5:526–542.

32. Rosse, W.F., Hillmen, P. and Schreiber, A.D. (2004) Immune-mediated hemolytic anemia. Hematology Am Soc Hematol Educ Program 2004:48-62.

33. Barratt, J., Smith, A.C. and Feehally, J. (2007) The pathogenic role of IgA1 O-glycosylation in the pathogenesis of IgA nephropathy. Nephrology (Carlton) 12:275–284.

34. Piller, F., Le Deist, F., Weinberg, K.I., Parkman, R. and Fukuda, M. (1991) Altered O-glycan synthesis in lymphocytes from patients with Wiskott-Aldrich syndrome. J Exp Med 173:1501-10.

35. Yu, H. and Chen, X. (2007) Carbohydrate post-glycosylational modifications. Org Biomol Chem 5:865-872.

36. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3:97-130.

37. Lee, Y.C. (1992) Biochemistry of carbohydrate-protein interaction. FASEB J 6:3193-3200.

38. Kim, Y.J. and Varki, A. (1997) Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconj J 14, 569-576.

39. Gornik, I., Maravic, G., Dumic, J., Flogel, M., and Lauc, G. (1999) Fucosylation of IgG heavy chains is increased in rheumatoid arthritis. Clin Biochem 32, 605-608.

40. Goodarzi, M.T., Axford, J.S., Varanasi, S.S., Alavi, A., Cunnane, G., Fitzgerald, O. and Turner, G.A. (1998) Sialyl Lewis(x) expression on IgG in rheumatoid arthritis and other arthritic conditions: a preliminary study. Glycoconj J 15:1149-1154.

41. Etzioni, A., Frydman, M., Pollack, S., Avidor, I., Phillips, M.L., Paulson, J.C. and Gershoni-Baruch, R. (1992) Brief report: recurrent severe infections caused by a novel leukocyte adhesion deficiency. N Engl J Med 327:1789–1792.

42. Becker, D.J. and Lowe, J.B. (1999) Leukocyte adhesion deficiency type II. Biochim Biophys Acta 1455:193-204.

43. Smith, P.L., Myers, J.T., Rogers, C.E., Zhou, L., Petryniak, B., Becker, D.J., Homeister, J.W. and Lowe, J.B. (2002) Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. J Cell Biol 158:801-15.

44. Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R. and Aguet, M. (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 10:547-558.

45. Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002) Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. Int Immunol 14:637-645.

46. Weng, A.P., Ferrando, A.A., Lee, W., Morris, J.P. 4th, Silvermanm, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T. and Aster, J.C. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306:269-71.

47. Chiang, M.Y., Xu, L., Shestova, O., Histen, G., L'heureux, S., Romany, C., Childs, M.E., Gimotty, P.A., Aster, J.C. and Pear, W.S. (2008) Leukemia-associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. J Clin Invest 118:3181-3194.

48. Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E. M., Rand, E. B., Piccoli, D. A., Hood, L. and Spinner, N. B. (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nature Genet 16: 243-251.

49. McDaniell, R., Warthen, D. M., Sanchez-Lara, P. A., Pai, A., Krantz, I. D., Piccoli, D. A. and Spinner, N. B. (2006) NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the Notch signaling pathway. Am J Hum Genet 79: 169-173.

50. Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E., Maclazek, J., Vayssiere, C., Cruaud, C., Cabanis, E.-A., Ruchoux, M. M., Weissenbach, J., Bach, J. F., Bousser, M. G. and Tournier-Lasserve, E. (1996) Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 383: 707-710.

51. Garg, V., Muth, A. N., Ransom, J. F., Schluterman, M. K., Barnes, R., King, I. N., Grossfeld, P. D. and Srivastava, D. (2005) Mutations in NOTCH1 cause aortic valve disease. Nature 437: 270-274.

52. Eldadah, Z. A., Hamosh, A., Biery, N. J., Montgomery, R. A., Duke, M., Elkins, R. and Dietz, H.C. (2001) Familial tetralogy of Fallot caused by mutation in the jagged1 gene. Hum Molec Genet 10: 163-169.

53. Wharton, K.A., Johansen, K.M., Xu, T. and Artavanis-Tsakonas, S. (1985) Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43:567-581.

54. Shao, L., Luo, Y., Moloney, D.J. and Haltiwanger, R. (2002) O-glycosylation of EGF repeats: identification and initial characterization of a UDP-glucose: protein O-glucosyltransferase. Glycobiology 12:763-770.

55. Shao, L., Moloney, D.J. and Haltiwanger, R. (2003) Fringe modifies O-fucose on mouse Notch1 at epidermal growth factor-like repeats within the ligand-binding site and the Abruptex region. J Biol Chem 278:7775-7782.

56. Okajima, T. and Irvine, K.D. (2002) Regulation of notch signaling by O-fucose. Cell 111:893-904.

57. Okajima, T., Xu, A., Lei, L. and Irvine, K.D. (2006) Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. Science 307:1599-603.

58. Shi, S. and Stanley, P. (2003) Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proc Natl Acad Sci USA 100:5234-9.

59. Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K.D., Haltiwanger, R.S. and Vogt, T.F. (2000) Fringe is a glycosyltransferase that modifies Notch. Nature 406:369-75.

60. Hicks, C., Johnston, S.H., diSibio, G., Collazo, A., Vogt, T.F. and Weinmaster, G. (2000) Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. Nat Cell Biol 2:515-20.

61. Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Mönch, K., Astrand-Grundström, I., Sitnicka, E., Sasaki, Y. and Jacobsen, S.E. (2001) Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659-69.

62. Sieff, C.A., Nathan, D.G. and Clark, S.C. (2003) The Anatomy and Physiology of Hematopoiesis. In D.G. Nathan, S.H., Orkin, D. Ginsburg and A.T. Look (Eds.) Nathan and Oski's Hematology of Infancy and Childhood 6th Edition (pp. 172-255). Philadelphia: W.B. Saunders Company.

63. Orkin, S.H. and Zon, L.I. (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132:631-44.

64. Rajasagi, M., Vitacolonna, M., Benjak, B., Marhaba, R. and Zöller, M. (2009) CD44 promotes progenitor homing into the thymus and T cell maturation. J Leukoc Biol 85:251-261.

65. Benz, C., Heinzel, K., and Bleul, C.C. (2004) Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. Eur J Immunol 34:3652-3663.

66. Rossi, F.M., Corbel, S.Y., Merzaban, J.S., Carlow, D.A., Gossens, K., Duenas, J., So, L., Yi, L. and Ziltener, H.J. (2005) Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nat Immunol 6:626-634.

67. Terra, R., Louis, I., Le Blanc, R., Ouellet, S., Zúñiga-Pflücker, J.C. and Perreault, C. (2005) T-cell generation by lymph node resident progenitor cells. Blood 106:193-200.

68. Heinzel, K., Benz, C., Martins, V.C., Haidl, I.D. and Bleul, C.C. (2007) Bone Marrow-Derived Hemopoietic Precursors Commit to the T Cell Lineage Only after Arrival in the Thymic Microenvironment. J Immunol 178, 858-868

69. Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N.R., Duarte, A., Macdonald, H.R. and Radtke, F. (2008) Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med 205:2515-2523.

70. Doefler, P., Shearman, M.S., and Perlmutter, R.M. (2001). Presenilin-dependent γ-secretase activity modulates thymocyte development. Proc Natl Acad Sci USA 98:9312-9317.

71. Pui, J.C., Allman, D., Xu, L., DeRocco, S., Karnell, F.G., Bakkour, S., Lee, J.Y., Kadesch, T., Hardy, R.R. Aster, J.C. and Pear, W.S. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 11:299-308.

72. Allman, D., Punt, J.A., Izon, D.J., Aster, J.C. and Pear, W.S. (2002) An invitation to T and more: notch signaling in lymphopoiesis. Cell 109 Suppl:S1-11.

73. Milner, L.A, Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I.D. and Martin, D.I. (1996) Inhibition of granulocytic differentiation by mNotch1. Proc Natl Acad Sci U S A 93:13014-13019.

74. Bigas, A., Martin, D.I. and Milner, L.A. (1998) Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol 18:2324-2333.

75. Kawamata, S., Du, C., Li, K. and Lavau, C. (2002) Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations. Oncogene 21:3855-3863.

76. de Pooter, R.F., Schmitt, T.M., de la Pompa, J.L., Fujiwara, Y., Orkin, S.H. and Zúñiga-Pflücker, J.C. (2006) Notch signaling requires GATA-2 to inhibit

myelopoiesis from embryonic stem cells and primary hemopoietic progenitors. J Immunol 176:5267-5275.

77. Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D.T. (2002) Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 99:2369-2378.

78. Kim, Y.W., Koo, B.K., Jeong, H.W., Yoon, M.J., Song, R., Shin, J., Jeong, D.C., Kim, S.H. and Kong, Y.Y. (2008) Defective Notch activation in microenvironment leads to myeloproliferative disease. Blood 112:4628-2638.

79. Schroeder, T. and Just, U. (2000) Notch signalling via RBP-J promotes myeloid differentiation. EMBO J 19:2558-2568.

80. Tan-Pertel, H.T., Walker, L., Browning, D., Miyamoto, A., Weinmaster, G. and Gasson, J.C. (2000) Notch signaling enhances survival and alters differentiation of 32D myeloblasts. J Immunol 165:4428-4436.

81. Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., MacDonald, H.R. and Radtke, F. (2005) Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood 105:2340-2342.

82. Aster, J.C., Pear, W.S. and Blacklow, S.C. (2008) Notch signaling in leukemia. Annu Rev Pathol Mech Dis 3:587-613.

83. Anderson, G. and Jenkinson, E.J. (2001) Lymphostromal interactions in thymic development and function. Nat Rev Immunol 1:31-40.

84. Becker, D.J. and Lowe, J.B. (2003) Fucose: biosynthesis and biological function in mammals. Glycobiology 13:41R-53R.

85. Maillard, I., Fang, T. and Pear, W.S. (2005) Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu Rev Immunol 23:945-974.

86. Zhou, L., Li, L.W., Yan, Q., Petryniak, B., Man, Y., Su, C., Shim, J., Chervin, S. and Lowe, J.B. (2008) Notch-dependent control of myelopoiesis is regulated by fucosylation. Blood 112:308-19.

87. Wang, Y. and Spellman, M.W. (1998) Purification and Characterization of a GDP-fucose:Polypeptide Fucosyltransferase from Chinese Hamster Ovary Cells. J Biol Chem 273:8112–8118.

88. Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P. and Haltiwanger, R.S. (2001) Modification of epidermal growth factor-like repeats

with O-fucose: molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. J Biol Chem 276:40338-40345.

89. Conlon, R.A., Reaume, A.G. and Rossant, J. (1995) Notch1 is required for the coordinate segmentation of somites. Development 121:1533-45.

90. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R.S. and Panin, V.M. (2006) Protein O-fucosyltransferase 2 adds O-fucose to thrombospondin type 1 repeats. J Biol Chem 281:9393-9399.

91. Schmitt, T.M. and Zuniga-Pflucker, J.C. (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17:749-756.

92. Scimone, M.L., Aifantis, I., Apostolou, I., von Boehmer, H. and von Andrian, U.H. (2006) A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. Proc Natl Acad Sci U S A 103:7006-11.

93. Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S. and Zúñiga-Pflücker, J.C. (2004) Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol 5:410-417.

94. Schiffer, S.G., Foley, S., Kaffashan, A., Hronowski, X., Zichittella, A.E., Yeo, C.Y., Miatkowski, K., Adkins, H.B., Damon, B., Whitman, M., Salomon, D., Sanicola, M. and Williams, K.P. (2001) Fucosylation of Cripto is required for its ability to facilitate nodal signaling. J Biol Chem 276:37769-37778.

95. Yan, Y.T., Liu, J.J., Luo, Y., Haltiwanger, R.S., Abate-Shen, C. and Shen, M.M. (2002) Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol Cell Biol 22:4439-4449.

96. Harris, R.J., Ling, V.T. and Spellman, M.W. (1992) O-fucose is present in the first epidermal growth factor domain of factor XII but not protein C. J Biol Chem 267:5102-5107.

97. Stahl, M., Uemura, K., Ge, C., Shi, S., Tashima, Y. and Stanley, P. (2008) Roles of Pofut1 and O-fucose in mammalian Notch signaling. J Biol Chem 283:13638-13651.

98. Laiosa, C.V., Stadtfeld, M., Xie, H., de Andres-Aguayo, L. and Graf, T. (2006) Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. Immunity 25:731-744.

99. Sitnicka, E., Bryder, D., Theilgaard-Mönch, K., Buza-Vidas, N., Adolfsson, J. and Jacobsen, S.E. (2002) Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. Immunity 17:463-72.

100. Friedman, A.D. (2002) Transcriptional regulation of granulocyte and monocyte development. Oncogene 21:3377-3390.

101. Anderson, M.K., Weiss, A.H., Hernandez-Hoyos, G., Dionne, C.J. and Rothenberg, E.V. (2002) Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T cell development at the pro-T cell stage. Immunity 16:285-296.

102. Sakata-Yanagimoto, M., Nakagami-Yamaguchi, E., Saito, T., Kumano, K., Yasutomo, K., Ogawa, S., Kurokawa, M. and Chiba, S. (2008) Coordinated regulation of transcription factors through Notch2 is an important mediator of mast cell fate. Proc Natl Acad Sci U S A 105:7839-7844.

103. Hozumi, K., Negishi, N., Suzuki, D., Abe, N., Sotomaru, Y., Tamaoki, N., Mailhos, C., Ish-Horowicz, D., Habu, S. and Owen, M.J. (2004) Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat Immunol 5:638-644.

104. Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K., Nakagami-Yamaguchi, E., Hamada, Y., Aizawa, S. and Hirai, H. (2003) Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity 18:675–685.

105. Zhang, N. and Gridley, T. (1998) Defects in somite formation in lunatic fringe-deficient mice. Nature 394:374-377.

106. Evrard, Y.A., Lun, Y., Aulehla, A., Gan, L. and Johnson, R.L. (1998) lunatic fringe is an essential mediator of somite segmentation and patterning. Nature 394:377-381.

107. Visan, I., Yuan, J.S., Tan, J.B., Cretegny, K. and Guidos, C.J. (2006) Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions. Immunol Rev 209:76-94.

108. Tan, J.B., Xu, K., Cretegny, K., Visan, I., Yuan, J.S., Egan, S.E. and Guidos, C.J. (2009) Lunatic and manic fringe cooperatively enhance marginal zone B cell precursor competition for delta-like 1 in splenic endothelial niches. Immunity 30:254-263.

109. Maillard, I., Koch, U., Dumortier, A., Shestova, O., Xu, L., Sai, H., Pross, S.E., Aster, J.C., Bhandoola, A., Radtke, F. and Pear, W.S. (2008) Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell Stem Cell 2:356-366.

110. Yan, Q., Yao, D., Wei, L.L., Huang, Y., Myers, J., Zhang, L., Xin, W., Shim, J., Man, Y., Petryniak, B., Gerson, S., Lowe, J.B. and Zhou, L. (2010) O-fucose modulates notch-controlled blood lineage commitment. Am J Pathol 176:2921-2934.

111. Ge, C. and Stanley, P. (2008) The O-fucose glycan in the ligand-binding domain of Notch1 regulates embryogenesis and T cell development. Proc Natl Acad Sci U S A 105:1539-1544.

112. Ge, C., Liu, T., Hou, X. and Stanley, P. (2008) In vivo consequences of deleting EGF repeats 8-12 including the ligand binding domain of mouse Notch1. BMC Dev Biol 8:48.

113. Shi, S., Stahl, M., Lu, L. and Stanley, P. (2005) Canonical Notch signaling is dispensable for early cell fate specifications in mammals. Mol Cell Biol 25:9503-9508.

114. Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., Hamada, Y., Yazaki, Y. and Hirai, H. (1999) Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. J Biol Chem 274:32961-32969.

115. Kuhn, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995) Inducible gene targeting in mice. Science 269:1427-1429.

116. Hozumi, K., Mailhos, C., Negishi, N., Hirano, K., Yahata, T., Ando, K., Zuklys, S., Holländer, G.A., Shima, D.T. and Habu, S. (2008) Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med 205:2507-2513.

117. Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B.J., Cado, D. and Robey, E. (1997) Notch activity influences the alphabeta versus gammadelta T cell lineage decision. Cell 88:833-843.

118. Visan, I., Tan, J.B., Yuan, J.S., Harper, J.A., Koch, U. and Guidos, C.J. (2006) Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. Nat Immunol 7:634-643.

119. Panin, V.M., Shao, L., Lei, L., Moloney, D.J., Irvine, K.D. and Haltiwanger, R.S. (2002) Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. J Biol Chem 277:29945-29952.

120. Okajima, T., Xu, A. and Irvine, K.D. (2003) Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. J Biol Chem 278:42340-42345.

121. Röpke, A., Kujat, A., Gräber, M., Giannakudis, J. and Hansmann, I. (2003) Identification of 36 novel Jagged1 (JAG1) mutations in patients with Alagille syndrome. Hum Mutat 21:100.

122. Valtieri, M., Tweardy, D.J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D. and Rovera, G. (1987) Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative responses in a murine progenitor cell line. J Immunol 138:3829-2835.

123. Fortini, M.E. (2009) Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 16:633-647.

124. Aulehla, A. and Johnson, R.L. (1999) Dynamic expression of lunatic fringe suggests a link between notch signaling and an autonomous cellular oscillator driving somite segmentation. Dev Biol 207:49-61.

125. Rampal, R., Arboleda-Velasquez, J.F., Nita-Lazar, A., Kosik, K.S. and Haltiwanger, R.S. (2005) Highly conserved O-fucose sites have distinct effects on Notch1 function. J Biol Chem 280:32133-32140.

126. Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell 67:687-699.

127. Xu, A., Lei, L. and Irvine, K.D. (2005) Regions of Drosophila Notch that contribute to ligand binding and the modulatory influence of Fringe. J Biol Chem 280:30158-30165.

128. Varnum-Finney, B., Wu, L., Yu, M., Brashem-Stein, C., Staats, S., Flowers, D., Griffin, J.D. and Bernstein, I.D. (2000) Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J Cell Biol 113:4313-4318.

129. Rand, M.D., Grimm, L.M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S.C., Sklar, J. and Aster, J.C. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol 20:1825-1835.

130. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. and

Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398:518-522.

131. Crowe, R., Henrique, D., Ish-Horowicz, D. and Niswander, L. (1998) A new role for Notch and Delta in cell fate decisions: patterning the feather array. Development 125:767-775.

132. Villa, N., Walker, L., Lindsell, C.E., Gasson, J., Iruela-Arispe, M.L. and Weinmaster, G. (2001) Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev 108:161-164.

133. Shimizu, K., Chiba, S., Hosoya, N., Kumano, K., Saito, T., Kurokawa, M., Kanda, Y., Hamada, Y. and Hirai, H. (2000) Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. Mol Cell Biol 20:6913-6922.

134. Yang, L.T., Nichols, J.T., Yao, C., Manilay, J.O., Robey, E.A. and Weinmaster, G. (2005) Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. Mol Biol Cell 16:927-942.

135. Lei, L., Xu, A., Panin, V.M. and Irvine, K.D. (2003) An O-fucose site in the ligand binding domain inhibits Notch activation. Development 130:6411-6421.

136. de Celis, J.F. and Bray, S.J. (2000) The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. Development 127:1291-302.

137. Arboleda-Velasquez, J.F., Rampal, R., Fung, E., Darland, D.C., Liu, M., Martinez, M.C., Donahue, C.P., Navarro-Gonzalez, M.F., Libby, P., D'Amore, P.A., Aikawa, M., Haltiwanger, R.S. and Kosik, K.S. (2005) CADASIL mutations impair Notch3 glycosylation by Fringe. Hum Mol Genet 14:1631-1639.

138. Porritt, H.E., Rumfelt, L.L., Tabrizifard, S., Schmitt, T.M., Zuniga-Pflucker, J.C. and Petrie, H.T. (2004) Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity 20:735–45.

139. Lind, E.F., Prockop, S.E., Porritt, H.E. and Petrie, H.T. (2001) Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. J Exp Med 194:127–34.

140. Wolfer, A., Wilson, A., Nemir, M., MacDonald, H.R. and Radtke, F. (2002) Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. Immunity 16:869-879.

141. Tanigaki, K., Tsuji, M., Yamamoto, N., Han, H., Tsukada, J., Inoue, H., Kubo, M. and Honjo, T. (2004) Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. Immunity 20:611–622.

142. Jiang, R., Lan, Y., Chapman, H.D., Shawber, C., Norton, C.R., Serreze, D.V., Weinmaster, G., and Gridley, T. (1998) Defects in limb craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev 12:1046-1057.

143. Van de Walle, I., De Smet, G., De Smedt, M., Vandekerckhove, B., Leclercq, G., Plum, J. and Taghon, T. (2009) An early decrease in Notch activation is required for human TCR-alphabeta lineage differentiation at the expense of TCR-gammadelta T cells. Blood 113:2988-2998.

144. Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. (1996) An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell 87:483-492.

145. Fowlkes, B.J. and Robey, E.A. (2002) A reassessment of the effect of activated Notch1 on CD4 and CD8 T cell development. J Immunol 169:1817-1821.

146. Wolfer, A., Bakker, T., Wilson, A., Nicolas, M., Ioannidis, V., Littman, D.R., Wilson, C.B., Held, W., MacDonald, H.R. and Radtke, F. (2001) Inactivation of Notch1 in immature thymocytes does not perturb CD4 or CD8 T cell development. Nat Immunol 2:235-241.

147. Adler, S.H., Chiffoleau, E., Xu, L., Dalton, N.M., Burg, J.M., Wells, A.D., Wolfe, M.S., Turka, L.A. and Pear, W.S. (2003) Notch signaling augments T cell responsiveness by enhancing CD25 expression. J. Immunol 171:2896–903.

148. Palaga, T., Miele, L., Golde, T. E. and Osborne, B.A. (2003) TCR-mediated Notch signaling regulates proliferation and IFN- production in peripheral T cells. J Immunol 171:3019–3024.

149. Radtke, F., Wilson, A., Ernst, B. and MacDonald, H.R. (2002) The role of Notch signaling during hematopoietic lineage commitment. Immunol Rev 187:65–74.

150. Eagar, T.N., Tang, Q., Wolfe, M., He, Y., Pear, W.S. and Bluestone, J.A. (2004) Notch 1 signaling regulates peripheral T cell activation. Immunity 20:407-415.

151. Maekawa, Y., Tsukumo, S., Chiba, S., Hirai, H., Hayashi, Y., Okada, H., Kishihara, K. and Yasutomo, K. (2003) Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. Immunity 19:549-559.

152. Amsen, D., Blander, J.M., Lee, G.R., Tanigaki, K., Honjo, T. and Flavell, R.A. (2004) Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell 117:515-526.

153. Flynn, D.M., Nijjar, S., Hubscher, S.G., de Goyet Jde, V., Kelly, D.A., Strain, A.J. and Crosby, H.A. (2004) The role of Notch receptor expression in bile duct development and disease. J Pathol 204:55-64.

154. Loomes, K.M., Taichman, D.B., Glover, C.L., Williams, P.T., Markowitz, J.E., Piccoli, D.A., Baldwin, H.S. and Oakey, R.J. (2002) Characterization of Notch receptor expression in the developing mammalian heart and liver. Am J Med Genet 112:181-189.

155. Felli, M.P., Maroder, M., Mitsiadis, T.A., Campese, A.F., Bellavia, D., Vacca, A., Mann, R.S., Frati, L., Lendahl, U., Gulino, A. and Screpanti, I. (1999) Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. Int Immunol 11: 1017-1025.

156. Taghon, T., M. Yui, A., Pant, R., Diamond, R.A., and Rothenberg, E.V. (2006) Developmental and molecular characterization of emerging β- and -selected pre-T cells in the adult mouse thymus. Immunity 24: 53-64.

157. Wang, W. and Malcolm, B.A. (2002) Two-stage polymerase chain reaction protocol allowing introduction of multiple mutations, deletions, and insertions, using QuikChange site-directed mutagenesis. Methods Mol Biol 182:37-43.

158. Olson, E.N., Arnold, H.H., Rigby, P.W. and Wold, B.J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell 85:1-4.

159. Van Seventer, G.A., Shimizu, Y., Horgan, K.J. and Shaw, S. (1990) The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J Immunol 144:4579-4586.

160. Yang, S.Y., Denning, S.M., Mizuno, S., Dupont, B. and Haynes, B.F. (1988) A novel activation pathway for mature thymocytes. Costimulation of CD2

(T,p50) and CD28 (T,p44) induces autocrine interleukin 2/interleukin 2 receptor-mediated cell proliferation. J Exp Med 168:1457-1468.

161. De Smedt, T., Smith, J., Baum, P., Fanslow, W., Butz, E. and Maliszewski, C.(2002) Ox40 costimulation enhances the development of T cell responses induced by dendritic cells in vivo. J Immunol 168:661-670.

162. Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A., Gosselin, M., Owen, L.R., Rudd, C.E. and Gutierrez-Ramos, J.C. (2000) The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 13:95-105.

163. Ginsburg, V. (1961) Studies on the biosynthesis of guanosine diphosphate L-fucose. J Biol Chem 236:2389-2393.

164. Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N. and Reya, T. (2005) Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 6:314-22.

165. Kunisato, A., Chiba, S., Nakagami-Yamaguchi, E., Kumano, K., Saito, T., Masuda, S., Yamaguchi, T., Osawa, M., Kageyama, R., Nakauchi, H., Nishikawa, M. and Hirai, H. (2003) HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. Blood 101:1777-1783.

166. Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M. and Scadden, D.T. (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425:841-846.

167. Zhan, Y. and Zhao, Y. (2008) Hematopoietic stem cell transplant in mice by intra-femoral injection. Methods Mol Biol 430:161-169.

168. Sackstein, R., Merzaban, J.S., Cain, D.W., Dagia, N.M., Spencer, J.A., Lin, C.P. and Wohlgemuth, R. (2008) Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. Nat Med 14:181-187.

169. Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S. and Chandrasekharappa, S.C. (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 16:235-242.

170. Bousso, P., Bhakta, N.R., Lewis, R.S. and Robey, E. (2002) Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. Science 296:1876-1880.

171. DeHart, S.L., Heikens, M.J. and Tsai, S. (2005) Jagged2 promotes the development of natural killer cells and the establishment of functional natural killer cell lines. Blood 105:3521-3527.

172. Watarai, H., Rybouchkin, A., Hongo, N., Nagata, Y., Sakata, S., Sekine, E., Dashtsoodol, N., Tashiro, T., Fujii, S., Shimizu, K., Mori, K., Masuda, K., Kawamoto, H., Koseki, H. and Taniguchi, M. (2010) Generation of functional NKT cells in vitro from embryonic stem cells bearing rearranged invariant Valpha14-Jalpha18 TCRalpha gene. Blood 115:230-237.

173. Samon, J.B., Champhekar, A., Minter, L.M., Telfer, J.C., Miele, L., Fauq, A., Das, P., Golde, T.E. and Osborne, B.A. (2008) Notch1 and TGFbeta1 cooperatively regulate Foxp3 expression and the maintenance of peripheral regulatory T cells. Blood 112:1813-1821.