Tuning Notch signals in T cell development

Sophie M. Lehar

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2005

Program Authorized to Offer Degree:
Immunology

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

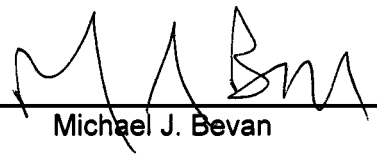
University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Sophie M. Lehar

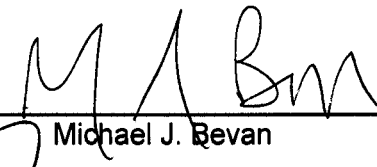
and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Chair of the Supervisory Committee:



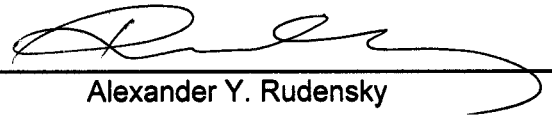
Michael J. Bevan

Reading Committee:



Michael J. Bevan

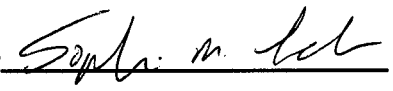
Christopher B. Wilson



Alexander Y. Rudensky

Date: NOV 29, 2005

In presenting this dissertation in partial fulfillment of the requirements for the doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to Proquest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346 (1-800-521-0600), to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature 

Date 11/29/05

University of Washington

Abstract

Tuning Notch signals in T cell development

Sophie M. Lehar

Chair of the Supervisory Committee:
Professor Michael J. Bevan

Department of Immunology

In this study, I have attempted to understand how Notch signals promote different stages of T cell maturation by examining the regulation of Notch signals on two levels. First, I have used an *in vitro* culture system to examine how differential signals through 2 classes of Notch ligands, Jagged and Delta, influence thymocyte differentiation. These data reveal that Notch signals inhibit B cell development and promote the maturation of immature thymocytes in two separable stages. While both classes of Notch ligands are able to inhibit B cell development, only Delta is able to promote the proliferation of immature thymocytes. Second, I have examined how Deltex, an intracellular modulator of Notch signals, regulates Notch signals by generating mice that are deficient in two of the three known Deltex homologues. Although there is considerable evidence that over-expression of Deltex in hematopoietic stem cells can inhibit Notch signals, and that Deltex is highly expressed in developing thymocytes, my data reveals that expression of Deltex in T cell progenitors is not essential for regulating the early stages of T cell maturation.

TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY

BM	Bone marrow.
BM-HSC	Bone marrow hematopoietic stem cell.
CBF1	Transcription factor that forms a critical component of the Notch. signaling pathway. Also known as RBPJκ or CSL.
CD4	Coreceptor molecule found on T-helper cells.
CD8	Coreceptor molecule found on cytotoxic T cells.
Cre	Recombinase that recognizes LoxP recognition sequences.
DC	Dendritic cell (the most important antigen presenting cell, necessary for the activation of naïve T cells).
DN	Double negative. Identifies a subset of immature thymocytes that do not express CD4 or CD8.
DP	Double positive. Identifies a population of immature thymocytes that express both CD4 and CD8.
ELP	Early lymphoid precursor.
ETP	Early thymic precursor.
FACS	Fluorescence-activated cell sorting.
FLP	Recombinase that recognizes FRT recognition sequences.
GFP	Green fluorescent protein.
LTRH	Retroviral expression vector used for expressing short interfering RNAs in conjunction with the human CD4 marker protein.
MHC-I	Class I Major histocompatiblitily antigen. Found on most cell types. Forms the basis of self-recognition by T cells. Binds to CD8 found on cytotoxic T cells.
MHC-II	Class II Major histocompatibility antigen. Expressed primarily on professional antigen presenting cells. Binds to CD4 found on T-helper cells.
MigR1	Retroviral vector used to express proteins in hematopoietic cells in conjunction with GFP.

NK	Natural Killer cell (lymphoid cell type that recognizes transformed and virally infected cells)
Notch-IC	Intracellular domain of the Notch receptor. Acts as a constitutively active receptor.
OP9	Stromal cell line derived from bone marrow. Supports B cell development in vitro.
Pre-Tα	Pre-T cell receptor alpha chain. Pairs with the TCRβ chain during early thymocyte development.
RAG	Recombination activating gene. Required for rearrangement of the genes encoding the T cell and B cell receptors.
RNAi	RNA interference. Method for inhibiting the expression of specific genes using short double stranded fragments of RNA.
RT-PCR	Reverse transcription polymerase chain reaction. Method for quantitating the amount of RNA.
SP	Single positive. Identifies the most mature population of thymocytes that express either CD4 or CD8.
TaqMan	Method for quantitative analysis of mRNA expression.
TCR	T cell receptor. 2 major classes include the αβ TCR found on most T-helper and cytotoxic T cells, and the more rare γδ TCR which defines a sublineage of mature T cells.
TEC	Thymic epithelial cell.

ACKNOWLEDGEMENTS

I would like to thank the University of Washington department of Immunology for all of their support over the last six years. My experience at UW has changed me in ways that extend well beyond the excellent scientific training I received. I specially want to thank Michael Bevan, for his clear thinking in science and his honest approach towards people. The road was not always easy on my project, and perhaps I hoped for better luck in my results. But, success in science depends on more than luck. When I first joined the lab, I would get a result and try to calculate how it fit into the model of immunology that I was building in my mind. Sometimes I would ask Mike what he thought, and more often than not, instead of lecturing me on his vision of Immunology, he would just answer with another question. Finally, I came to recognize that learning comes less from finding the answers, and more from asking the right questions. Mike has an amazing talent for gliding past all of the noise and zeroing in on what is missing, and what is most important. I hope that I have learned just a little bit of this insight from him as I move on in science.

I also want to thank all of my colleagues who overlapped with my stay in the Bevan lab. I have learned so much from all of their helpful comments as I struggled through my project, but a few have touched me most deeply. Particularly Eugene Huang, who guided me through my first few years in the Bevan lab. Eugene was a true inspiration, for his generosity, and I will always think of how much he helped me as I embark on a new writing project. I also want to thank Joke den Haan, who was always willing to listen to my ideas, and gave me such great support. On a personal level I want to thank the friends who stuck with me, and supported me through the ups and downs, especially my friends Richard Frazee, and Mark Ratner, who always seemed to have more confidence in me than I had in myself.

Chapter I: Introduction

Signals from different pathways must be integrated to regulate development.

Multicellular organisms develop from a single fertilized egg that must divide and differentiate to form the complex structures found in the adult. As an organism grows, each cell must find its way to the correct anatomical location, and then differentiate into the appropriate cell type. To do this effectively, each cell must constantly sense its environment to reaffirm who it is and where it is. Then, it must decide whether to live, die, divide or differentiate. The signals whereby cells make these important decisions are derived from a collection of signaling pathways that regulate the majority of cell-cell interactions. These include the receptor tyrosine kinase, JAK/STAT, Wnt, TGF-β, Hedgehog, and Notch receptor pathways[1,2]. Each of these signaling networks offers unique features that are best suited to different tasks. The specific requirements of the job at hand -such as whether signals must be transmitted rapidly, or gradually over large distances, equally across a region of cells or selectively through direct cell-cell contact- determine which pathway is the best choice. How all of the signals emanating from these different pathways are integrated to regulate the development of an organism is a central question in the study of biology.

Blood cells are derived from a common precursor.

The immune system is made up of a collection of different cell types that are specialized to perform specific tasks and can be broadly separated into the innate and adaptive immune systems. The innate immune system is made up primarily of cells of myeloid origin which recognize pathogens through inherited receptors that detect common determinants found on pathogens and form a primary line of defense. The adaptive immune system is made up of lymphoid cells, T cells and B cells, which express randomly generated receptors that do not have an inherent capacity to recognize pathogens, but must be selected by

prior exposure to antigen. The cells comprising these two major arms of the immune system cannot regenerate themselves and must differentiate from a self-renewing progenitor, the hematopoietic stem cell (HSC), which gives rise to all of the major hematopoietic lineages[3] (Figure1).

Hematopoietic stem cells comprise an extremely rare subset of cells found within the bone marrow that must continually replenish the correct mix of hematopoietic cells. This task is no simple matter because the total numbers of different cell types can vary dramatically. During early development, the first hematopoietic cells proliferate rapidly to populate an empty compartment. How do these early progenitors know when to start, and then stop dividing as the developing immune system is filled? Likewise, at the onset of infection specific cell types that are best suited to combat the infection at hand must expand, and then contract, after the infection is cleared. Throughout these processes, the total numbers of a given cell type can vary by as much as a thousand fold within a few days[4]. Throughout these changing conditions, a tiny subset of self-renewing stem cells that comprise less than 0.1% of the adult bone marrow provides a renewable source of progenitors. When mature lineages are depleted, these progenitors are somehow able to detect the need and respond by initiating their own maturation through a series of well-defined differentiative decisions. Throughout this process, stem cells, and their successively lineage-restricted progeny must constantly check their own progress against the current needs for each mature subset by continually interpreting signals derived from both intrinsic and extrinsic cues. Understanding how these signals are integrated is essential to our understanding of how the immune system functions in development, health and disease.

T cell and B cell development is parallel but distinct.

The adaptive immune system is made up of two major cell types B cells and T cells. B cells are specialized to produce soluble antibodies that neutralize pathogens by binding to their surface and tagging them for disposal and T cells

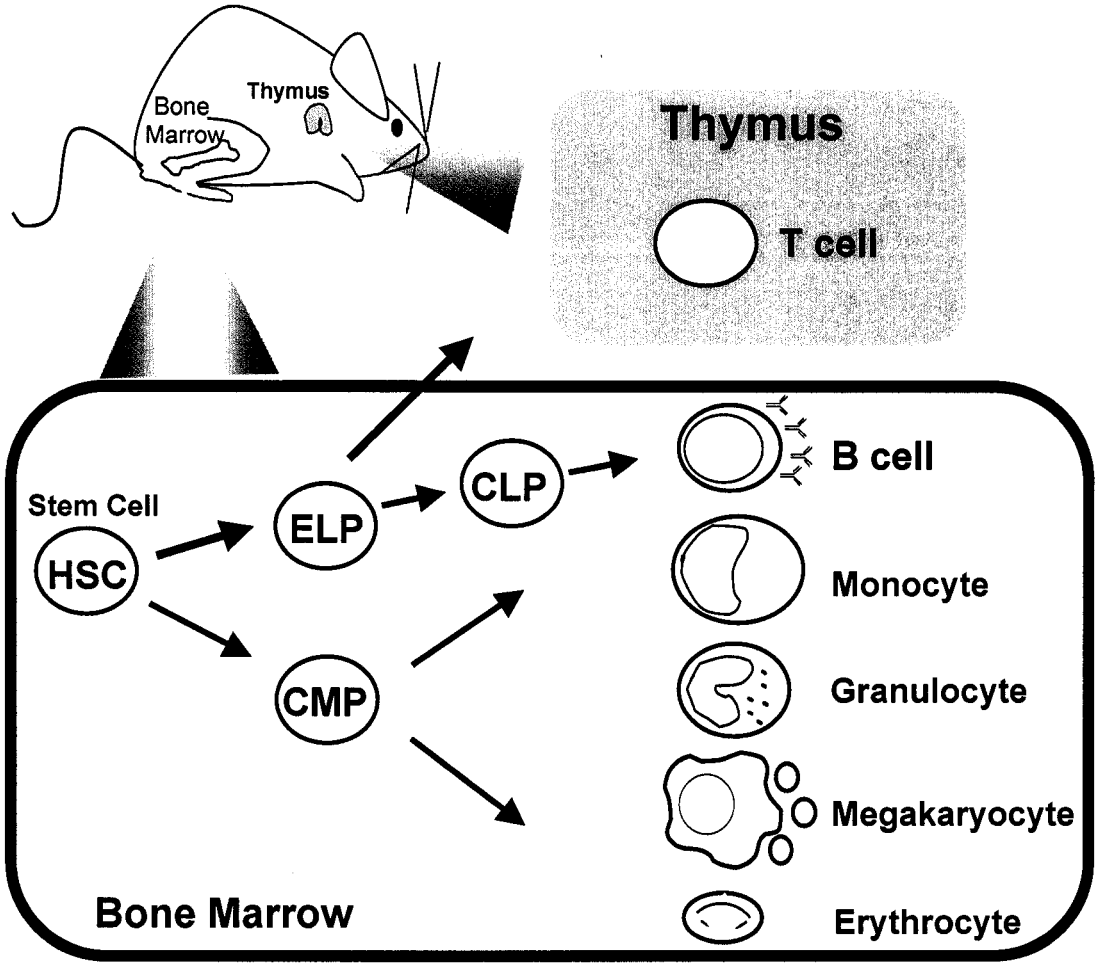


Figure 1: Blood cells are derived from HSC.

Hematopoietic cells are derived from a multipotent precursor (HSC) found in the bone marrow which undergoes a series of increasingly restrictive lineage choices to generate both lymphoid and myeloid cells. The innate immune system is made primarily of myeloid cells that derive from a common myeloid precursor (CMP). The adaptive immune system is made up of T cells and B cells that are derived from a common precursor, the early lymphoid precursor (ELP). T cells and B cells develop in different anatomical locations. While B cells develop within the bone marrow, T cell development occurs almost exclusively within the thymus.

perform important regulatory functions and provide cellular immunity. As they mature, B and T cell progenitors acquire the ability to recognize foreign invaders by expressing an antigen receptor on their cell surface: a membrane bound antibody for B cells or the T cell receptor (TCR) for T cells. While each lymphocyte expresses a single type of antigen receptor with unique specificity, lymphocyte populations contain an assortment of up to 10^9 different specificities[5], arming the organism with the capacity to recognize a near limitless collection foreign antigens. In both B and T cell development, this massive repertoire of antigen receptors is generated through a unique process of gene rearrangement that occurs only during defined stages of lymphocyte development. B cells and T cells develop from a common lymphoid precursor found in the bone marrow (Figure 1), and their respective progenitors pass through strikingly similar developmental stages as they sequentially rearrange first one, and then the other of the genes encoding the two chains of their antigen receptors. Although their development is remarkably similar, in the adult, T cells and B cells mature in distinct anatomical locations. While B cell development occurs primarily in the bone marrow T cell development occurs almost exclusively in the thymus. Hence, the signals regulating the development of these two types of cells must be both similar and different.

As B and T cell progenitors mature, their progress through distinct developmental stages is regulated by at least 3 different kinds of signals. First, signals derived from their newly generated antigen receptors provide a mechanism for monitoring each cell's ability to generate a functional antigen receptor with appropriate specificity[6,7]. Second, developing lymphocytes require specific growth factors such as interleukin 7 (IL-7) which is important for both B and T cell development[8,9]. These signals are transmitted through the JAK/STAT pathway and play an important role in regulating the overall number of lymphocytes that develop. A third signal directs the development of lymphocyte progenitors into the B *vs.* T cell lineages within the appropriate anatomical locations of the bone marrow or the thymus. A growing body of data reveals that this third signal is provided by stromal cells found within the organs where

lymphocyte development occurs, and these signals are transmitted largely through the Notch signaling pathway.

T cell progenitors progress through a series of developmental stages as they mature.

T cell development occurs primarily in the thymus. Within the thymus, T cell precursors must pass through a series of "check-points" that test their ability to express a functional T cell receptor. In the first step, bone marrow derived stem cells enter the thymus. These cells, termed early lymphoid precursors (ELP) comprise a poorly defined progenitor population that has been identified by a number of groups based on the expression of multiple cell surface molecules as lineage-marker negative, cKit^{hi}/Sca1^{hi} and IL7R^{neg}[10,11]. It is not clear whether ETPs represent a truly uncommitted lymphoid progenitor that commits to the T cell lineage in response to signals present in the thymus, or whether the thymus provides a permissive environment that is essential for the maturation of a pre-committed T cell progenitor. However, a rare population of cells that resemble thymic ETPs can be found in the bone marrow. These cells have been variably reported to express the Thy1[12] cell surface marker, or proposed to show early expression of the RAG-1[13] or pre-Tα genes[14].

The remaining stages of T cell maturation are closely tied to the sequential rearrangement of the genes encoding the two chains of the T cell receptor (TCR). During this process, T cell progenitors progress through a series of well defined maturational stages that can by monitored by the expression of a number of cell surface molecules including the CD4 and CD8 co-receptor molecules (Figure 2). The most immature thymocytes express neither CD4 nor CD8 and are termed double negative (DN). DN thymocytes can be further separated into four distinct developmental stages (DN1-DN4) defined by the expression of CD44 and CD25 surface molecules[15]. DN1 thymocytes (CD44$^+$/CD25$^-$) comprise a mixed population of precursors that retains the capacity to differentiate into T, B, NK and dendritic cells[16-18]. DN2 thymocytes

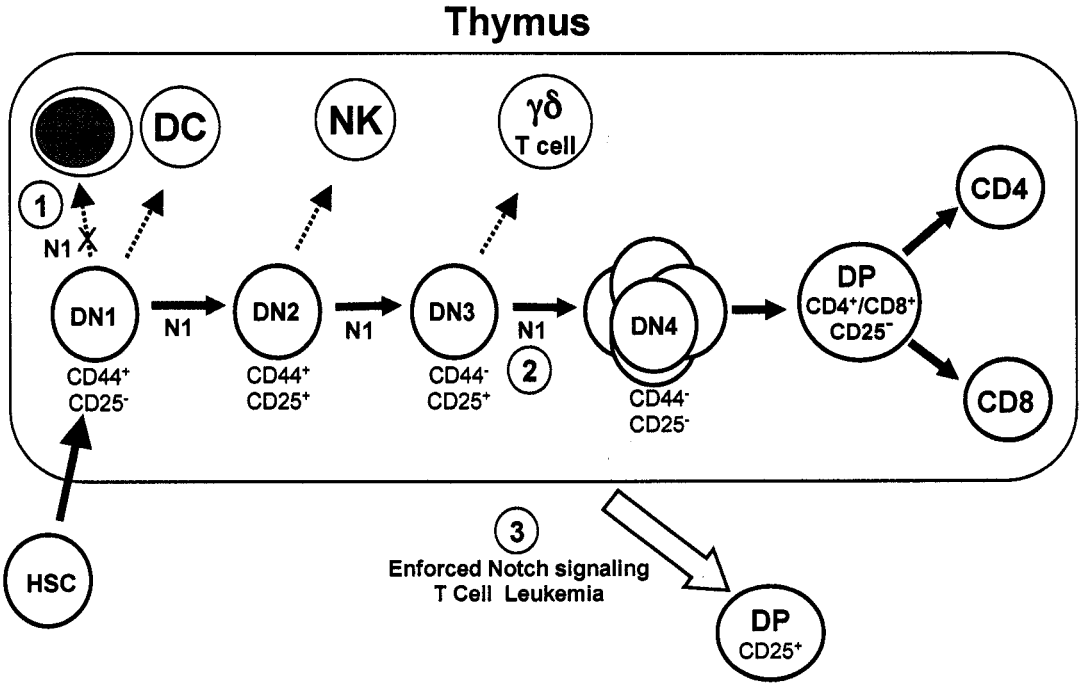


Figure 2: Notch signals regulate T cell maturation in the Thymus

Within the thymus, T cell progenitors pass through a series of developmental stages that can be monitored by their expression of cell surface molecules. The most immature thymocytes are termed double negative (DN) because they do not express either CD4 or CD8. DN thymocytes can be further separated into four distinct stages (DN1-DN4) by their expression of CD44 and CD25. DN1 thymocytes form a mixed population of uncommitted progenitors that retain the ability to differentiate into B cells, T cells, dendritic cells (DC) and natural killer cells (NK), while DN2 are restricted to the T or NK lineage. DN3 are committed to the T cell lineage. Gene rearrangement for the T cell receptor β, γ and δ chains occurs during the DN3 stage. Cells that successfully rearrange both TCRγ and TCRδ develop into the γδ T cell lineage, whereas those that express a functional TCRβ in conjunction with pre-Tα proliferate extensively and progress to the DP stage in a process termed β-selection. Notch signals regulate T cell development throughout the DN stages. (1) Notch signals inhibit B lineage commitment, and (2) continued Notch signaling is essential for the expansion of β-selected DN3 and DN4 thymocytes. Notch signaling is normally terminated during the double positive (DP) stage, and (3) excessive Notch signaling during this stage results in T cell leukemia.

($CD44^+/CD25^+$) lose the potential to differentiate into B cells but retain NK and dendritic cell potential[11,19], whereas DN3 thymocytes ($CD44^-/CD25^+$) are committed to the T cell lineage[20]. Thymocytes begin to rearrange the genes encoding TCRβ, TCRγ and TCRδ chains during the DN2/DN3 stage. During this stage, the majority of thymocytes that have successfully rearranged both TCRγ and TCRδ chain genes, differentiate into the γδ T cell lineage, whereas those that have successfully rearranged TCRβ express the pre-TCR consisting of TCRβ complexed with pre-Tα. The pre-TCR mediates β-selection, following which thymocytes undergo a massive proliferative expansion as they progress to the DN4 ($CD44^-/CD25^-$) stage. Only those cells that have undergone β-selection are able to upregulate CD4 and CD8 expression to enter the double positive (DP) stage[21-24], where they begin to rearrange the genes for TCRα and undergo positive selection prior to differentiating into either the CD4 or CD8 lineage[6,7,25,26].

T cell development depends on signals provided by the thymus.

Although the ability of T cell progenitors to progress through successive developmental stages is determined largely by their ability to express a functional T cell receptor, other signals are also important and it has long been known that T cell development requires signals that are provided by the stromal cells found in the thymus[27]. T cell progenitors migrate to specific regions within the thymus as they mature[28] suggesting that unique signals may be required for each successive developmental stage (Figure 3). The earliest T cell progenitors are thought to enter the thymus through blood vessels near the cortico-medullary junction, and migrate out toward the outer cortex as they begin to rearrange the genes for the TCRβ chain. The majority of proliferating DN3 and DN4 thymocytes that have undergone β-selection are found in the outer cortex. DP thymocytes undergo positive selection on cortical epithelial cells and the most mature CD4 or CD8 single positive thymocytes are found in the medulla, where they undergo negative selection to eliminate self-reactive T cells.

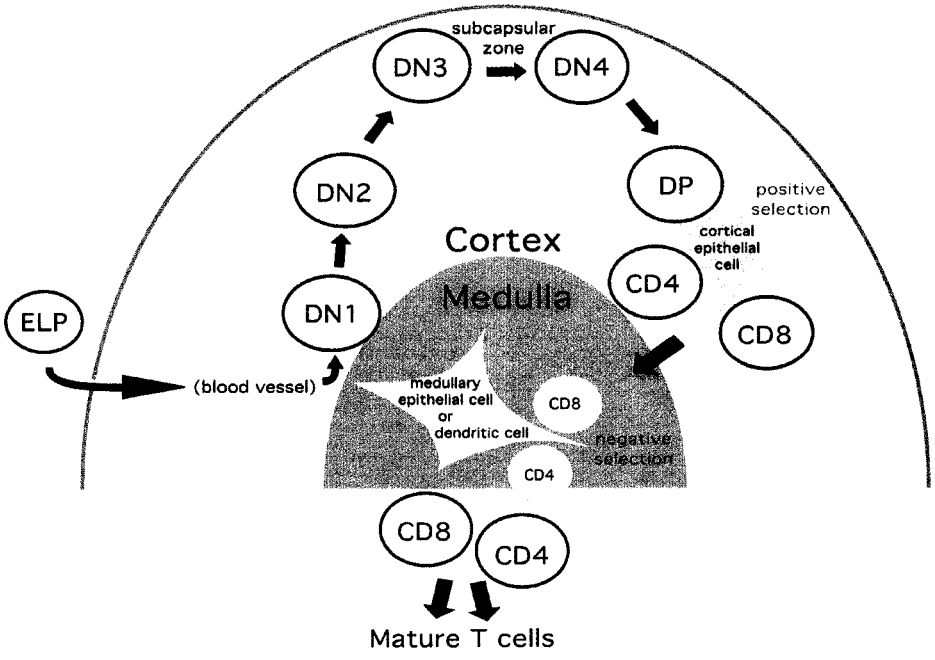


Figure 3: Migration of immature T cells in the thymus.
T cell progenitors migrate to distinct regions within the thymus as they mature. Lymphoid progenitors enter the thymus through blood vessels at the cortico-medullary junction, and migrate outward as they progress through the DN2 to DN3 stages. The majority of proliferating DN3 and DN4 thymocytes that have undergone β-selection are found in the outer cortex. DP thymocytes undergo positive selection on cortical epithelial cells, and the most mature CD4 or CD8 single positive thymocytes are found in the medulla, where they undergo negative selection to eliminate self-reactive T cells.

Efforts to understand the unique signals that allow the thymus to promote T cell development were initially stymied by the inability to induce T cell development *in vitro*. T cell maturation appeared to require some unknown signals that were only present in complex organ culture systems that recapitulated the 3-dimensional structure of the thymus. However, major progress in our understanding of these signals occurred a few years ago when a body of complementary data suggested that the unique signals provided by the thymic epithelium are derived largely through the Notch signaling pathway. This data revealed that in addition to signals through the TCR, Notch signals are essential for the earliest DN stages of T cell development to occur normally and can influence T cell maturation during almost every stage of their development.

The Notch signaling pathway

The Notch pathway is a conserved signaling mechanism that regulates cellular differentiation in a variety of tissue types throughout the life of multicellular organisms[29]. Notch signals are ideally suited to control the localized delivery of signals within the 3-dimensional architecture of organ systems, as both the ligands and receptors are membrane bound. Consequently, mutations in Notch ligands and receptors frequently result in severe developmental defects in a broad variety of tissues[30]. Notch signals have been shown to affect a range of cellular functions including proliferation[31,32], apoptosis[33,34] and developmental lineage choices[35-37].

In mammals there are four Notch receptors and five Notch ligands which signal through a common mechanism. Ligand binding induces a series of proteolytic cleavages within the Notch receptor. The terminal cleavage, which is mediated by presenilins, releases the intracellular domain of Notch (Notch-IC) which enters the nucleus and induces the transcription of Notch-responsive genes by binding to CBF1/RBPJκ, a transcriptional repressor/activator that is essential for the majority of Notch signal transduction[38] (Figure 4).

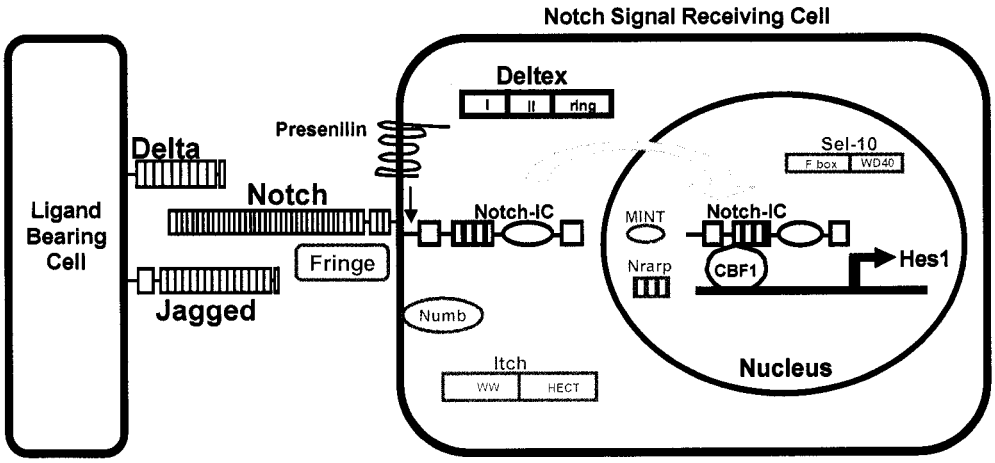


Figure 4: The Notch signaling pathway

Two classes of Notch ligands, Jagged and Delta, bind to Notch receptors. Ligand binding induces a presenilin-dependent proteolytic cleavage within the intracellular domain of Notch to generate the active form of Notch (Notch-IC). Notch-IC enters the nucleus where it binds to a DNA bound transcription factor CBF1. Notch signals are regulated at multiple levels. At the level of ligand binding, Fringe permits signaling through Delta and inhibits signaling through Jagged. Numb and Itch cooperate to regulate the activity of Notch-IC within the cytoplasm. Nrarp, MINT and Sel-10 regulate Notch signals within the nucleus. Deltex is found within the cytoplasm and nucleus and has been proposed to act as either a positive or negative regulator of Notch signals.

Notch ligands fall into two evolutionarily conserved classes: Delta (Delta 1,3 and 4) and Jagged (Jagged1 and Jagged2). The Delta and Jagged families encode related transmembrane proteins that share a conserved DSL (Delta/Serrate/Lag-2) domain that is required for binding to Notch, but they differ significantly in the overall size of their extracellular domains. Jagged molecules possess additional epidermal growth factor (EGF)-like repeats (16 rather than 8) and a unique cysteine-rich domain of unknown function[39].

The intensity or quality of Notch signals delivered to cells is finely tuned by a number of molecules that can regulate Notch signals on multiple levels[40] (Figure 4). At the level of ligand binding, Notch signals are regulated by a family of glycosyl transferases (Lunatic, Manic and Radical Fringe), that affect signals delivered through the Delta *vs.* Jagged classes of Notch ligands by altering the glycosylation of Notch[41]. In the absence of Fringe, Jagged and Delta can activate Notch signals similarly[42-45]. Expression of fringe in the Notch signal receiving cell is thought to prevent signaling through Jagged ligands while permitting Notch signals transmitted through Delta.

Within the Notch signal receiving cell, a number of molecules regulate the transmission of Notch signals within the cytoplasm or nucleus[46]. Numb is a cytoplasmic protein that is asymmetrically distributed in the progeny of dividing cells, and can inhibit Notch signals by preventing the nuclear translocation of Notch-IC[47]. Numb acts in cooperation with Itch, a ubiquitin ligase, to target membrane-bound Notch for degradation[48]. Within the nucleus, Sel-10 targets Notch-IC for degradation through the PEST domain of Notch[46]. Nrarp, a small ankyrin repeat containing protein[49], and MINT[50] can inhibit the transactivation of Notch responsive genes by competing with Notch-IC for binding to CBF1. In addition to the above molecules, a number of genes have been identified as putative regulators of Notch signaling through mutational analysis in flies or other organisms, but their mechanism of action remains uncertain. *Deltex* encodes a putative E3-ubiquitin ligase that was originally proposed to act as a positive

regulator of Notch signals during the development of neural tissues in the fruit fly *Drosophila*.

The existence of a variety of evolutionarily conserved molecules that appear to regulate Notch signals at almost every level, from the recognition of different ligands to the transactivation of Notch responsive genes inside the nucleus, suggests that precise regulation of Notch signals is essential for normal development. However, it remains uncertain whether these molecules act primarily by regulating the dose or duration of Notch signals, or whether they participate in altering the quality of Notch signals, for example by influencing the activation of specific Notch responsive genes. Differential regulation of Notch signals through these regulatory molecules could influence whether all or only a subset of Notch responsive genes are induced in different cellular contexts, or alter the outcome of Notch signaling in different cells by integrating Notch signals with other signaling pathways.

Notch signals regulate T cell development at multiple stages.

Notch signals have been shown to be important during several stages of T cell development, including T lineage commitment and during the early DN stages of T cell maturation[51,52]. An essential role for Notch signaling in promoting T lineage commitment was shown several years ago using complementary approaches. W. Pear and colleagues showed that Notch signals promote T lineage commitment by demonstrating that bone marrow stem cells transduced with a retrovirus expressing a constitutively active form of Notch1 (Notch-IC) commit to the T cell lineage in the absence of normal thymus derived signals and differentiate into DP T cells in the bone marrow[53,54]. Complementary studies by Radtke *at al.* demonstrated that, in the absence of Notch1, stem cells cannot differentiate into T cells[55] and adopt the B cell fate in the thymus[56]. This data suggested that in addition to promoting T lineage commitment, Notch signals are necessary to prevent B cell development in the thymus. Further studies revealed that abnormal T cell development in stem cells expressing Notch-IC is dependent

on signals through the pre-TCR, revealing that Notch signals cooperate with TCR signals to promote T cell development[57,58].

Subsequent data demonstrated that Notch signals continue to support T cell development throughout the early DN stages of maturation, and that the dose of Notch signals delivered to immature thymocytes can influence their ability to mature into different T cell sublineages. Using a conditional allele of Notch1, Radtke's group revealed that Notch1 signals are essential for normal thymocyte development up to at least the DN2/DN3 stage [59], but dispensable beyond the DN3 stage[60]. However, studies examining the maturation of T cell progenitors in vitro suggested that thymocytes continue to depend on Notch signals (potentially through other Notch family members) for their proliferative expansion during the DN3 and DN4 stages[61-63]. Several other studies have also suggested that the dose of Notch signals delivered to immature thymocytes can influence the differentiation of immature thymocytes into the $\alpha\beta$ vs. $\gamma\delta$ T cell lineages[64,65], although the mechanism whereby Notch signals influence these decisions is less clear.

There is little doubt that Notch signals are essential for promoting T lineage commitment and continue to influence multiple stages of T cell maturation. However, it remains uncertain how Notch signals direct lineage commitment, survival or the proliferation of T cell progenitors at the molecular level. To date, the array of cellular functions that are known to be influenced by Notch signals cannot be directly linked to the induction of specific Notch responsive genes that would be presumed to act as the messengers. We have attempted to understand how Notch signals regulate T cell development by examining the regulation of Notch signals on two different levels: ligand binding, and through the intracellular regulator Deltex.

Chapter II: Notch ligands transmit distinct signals to T cells.

Notch signals have been shown to be important during several stages of T cell development, including T lineage commitment and during the early DN stages of T cell maturation[51,52]. Over-expression of a constitutively active form of Notch1 in HSC results in a complete inhibition of B cell development and the appearance of DP T cells in the bone marrow[54,66]. Correspondingly, targeted deletion of Notch1 in stem cells results in the inhibition of T cell development, and an absolute increase in the number of immature B cells within the thymus[55,56]. In addition to its role in promoting T lineage commitment, Notch signals have been shown to influence the development of $\alpha\beta$ *vs.* $\gamma\delta$ T cells[64,65], and continue to be important throughout the DN stages of thymocyte development. Targeted deletion of Notch1 during the DN2 to DN3 stage results in a partial arrest of thymocyte development at the DN3 stage[59], and DN3 thymocytes that have received a functional pre-TCR signal continue to require Notch signals to proliferate in vitro[62].

Notch ligands fall into two evolutionarily conserved classes: Delta (Delta 1,3 and 4) and Jagged (Jagged1 and Jagged2). Delta and Jagged ligands are thought to activate Notch signals similarly through the activation of CBF1/RBPJκ[42-45]. Nevertheless, there is evidence that signals transmitted through Delta or Jagged can differentially affect the Notch-expressing cell. A study by Jaleco *et al.*[67], examining the effects of Delta1 and Jagged1 on human $CD34^+$ stem cells, suggested that only Delta promotes T lineage commitment. However, because mRNAs for Delta1, Delta4, Jagged1 and Jagged2 can be detected in the thymus[68-70], it is likely that T cell precursors encounter both classes of Notch ligands as they mature. It is therefore key to understand whether Notch signals through Delta and Jagged have distinct roles in regulating T cell development. To address this question, we have adapted a recently described in vitro culture system[68] to examine the effect of Delta1 *vs.* Jagged1 on T cell progenitors at specific developmental stages.

Immunohistochemical analysis of Jagged1 expression in the thymus.

Notch ligands belonging to both the Delta and Jagged classes have been shown, by PCR analysis, to be expressed in the thymus[68-70]. However, previous data suggested that signals from the Jagged class of Notch ligands are unable to promote T cell development[67]. To determine whether thymocytes are likely to encounter Jagged signals, we examined the spatial organization of Notch ligands within the adult thymus by immunohistochemistry. We were unable to obtain specific staining with antibodies for Delta1 or Jagged2 (see Methods). Results using antisera against Jagged1, for which staining was completely blocked by pre-incubation with the immunizing peptide (not shown), are shown in Figure 5. To differentiate between expression within the cortex or medulla, thymus sections were co-stained with antibodies ER-TR5[71] or CDR-1[72] which label the medullary or cortical epithelial cells respectively. Jagged1 is most highly expressed within the medulla, and co-staining with ER-TR5 reveals almost complete overlap of the two markers (Figure 5A, left panels). In the thymus cortex, Jagged1 is expressed on only a subset of cortical epithelial cells that form a loose network extending from the medulla to the capsule (Figure 5A, right panels).

Generation of OP9-Jagged1 stromal cells.

Forced expression of Delta1 on the bone marrow-derived stromal cell line OP9, that otherwise promotes only myeloid and B cell development, renders these cells able to induce robust proliferation and maturation of T cell precursors, up to at least the DP stage of development[68]. To compare Delta vs. Jagged signals, we over-expressed the full length cDNA for murine Jagged1 in the parental OP9 using the same expression vector (MigR1) that was used to express Delta1 in these cells. This vector allows co-expression of either Delta1 or Jagged1 as a bi-cistronic message with green fluorescence protein (GFP).

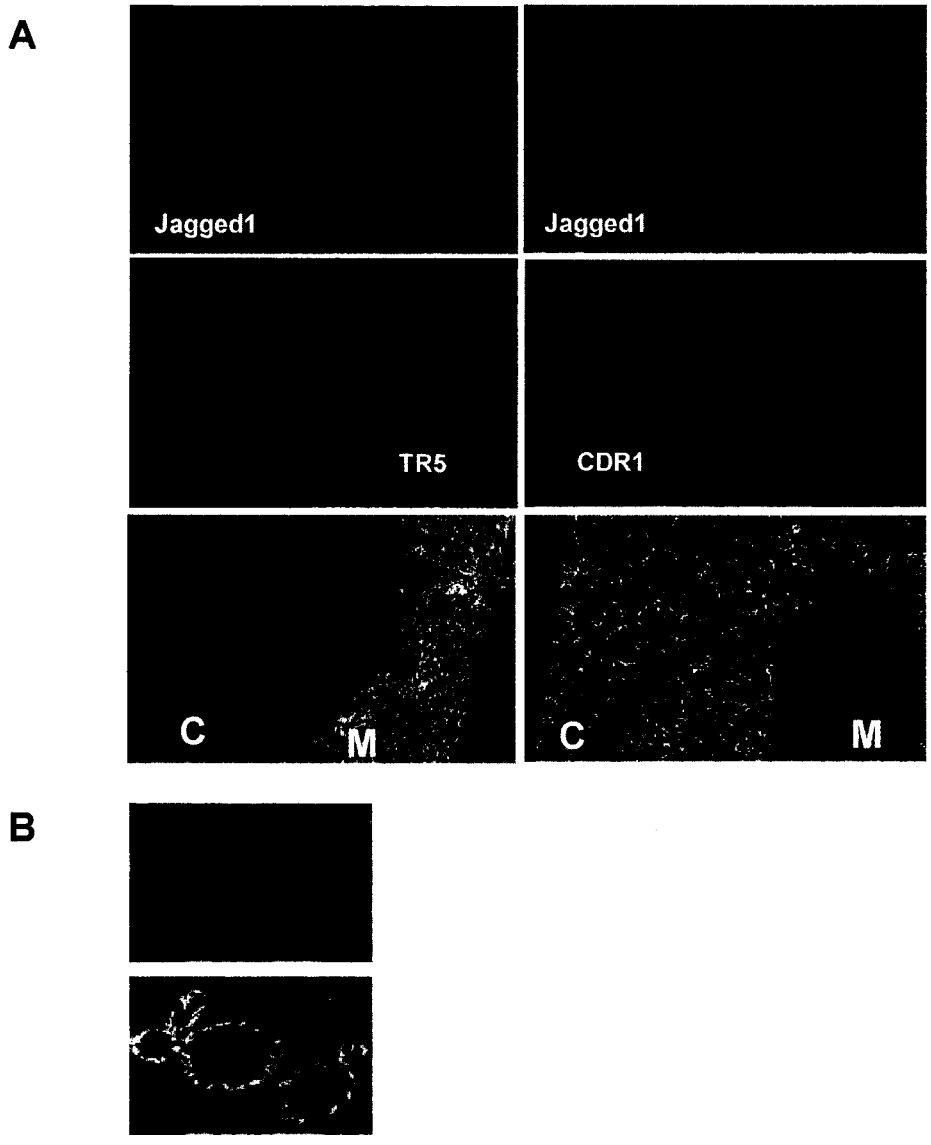


Figure 5: Jagged1 is expressed on thymic epithelial cells (TEC) and on the endothelial cells that form thymic blood vessels.

(A) Immunohistochemical analysis of Jagged1 expression in the thymus. Frozen sections of C57BL/6 thymic lobes were stained with antibody specific for the intracellular domain of Jagged1 (green) and co-stained (red) with antibodies specific for medullary (ER-TR5) or cortical epithelial cells (CDR1). The cortex (C) and medulla (M) are indicated in white (20X magnification).
(B) Magnification (40X) of a thymic blood vessel similar to that shown in A, co-stained for Jagged1 (green) and MEC (red) a blood vessel marker. Bottom panel shows an overlay of the two stains.

The resulting cell lines were sorted for expression of GFP, and the similar level of GFP fluorescence in OP9-MigR1, OP9-Jagged1 and OP9-Delta1[68], is shown in Figure 6C.

We examined the expression of the mRNAs for various Notch ligands in the OP9 cell lines and in the total thymus epithelial cell fraction (TEC) by RT-PCR analysis (Figure 6A). TEC express detectable levels of Jagged1, Jagged2 and Delta4 mRNAs. The parental OP9 stromal cells express low levels of Jagged1 and undetectable levels of Jagged2, Delta1 or Delta4 mRNAs. As expected, Jagged1 and Delta1 mRNAs are highly expressed in OP9-Jagged1 and OP9-Delta1 respectively. Surprisingly, expression of the endogenous Jagged1 mRNA is induced by approximately 25-fold in OP9-Delta1, suggesting that OP9-Delta1 could express both Delta1 and Jagged1 proteins. Therefore, we examined Jagged1 protein expression by immunoprecipitation followed by Western Blot. By this analysis, we were unable to detect endogenous Jagged1 in the parental OP9 cells, and only trace levels in OP9-Delta1 (Figure 6B). We confirmed that Jagged1 is expressed on the surface of OP9-Jagged1 cells by flow cytometric analysis using an antibody directed against the extracellular domain of Jagged1 (Figure 6C). The antibody gave similar levels of background staining on OP9-MigR1 and OP9-Delta1 cells, but a clear shift in staining on OP9-Jagged1 cells.

To ensure that Jagged1 is functional in the stromal cells and to directly compare the ability of OP9-Jagged1 and OP9-Delta1 to transmit Notch signals, we tested the cells in a well-characterized system in which Notch signals prevent the differentiation of C2C12 myoblast cells into mature myotubes[73]. In this system, C2C12 differentiation can be monitored either by the appearance of large multinucleated myotubes, or by the induction of myotube-specific genes such as myosin light chain (MLC2). Figure 7 shows that OP9 expressing Jagged1 or Delta1 both inhibit C2C12 differentiation, whereas OP9-MigR1 cells do not.

To confirm that the overall level of Notch signals delivered by OP9-Jagged1 and OP9-Delta1 were equivalent, we titrated the level of Notch signals

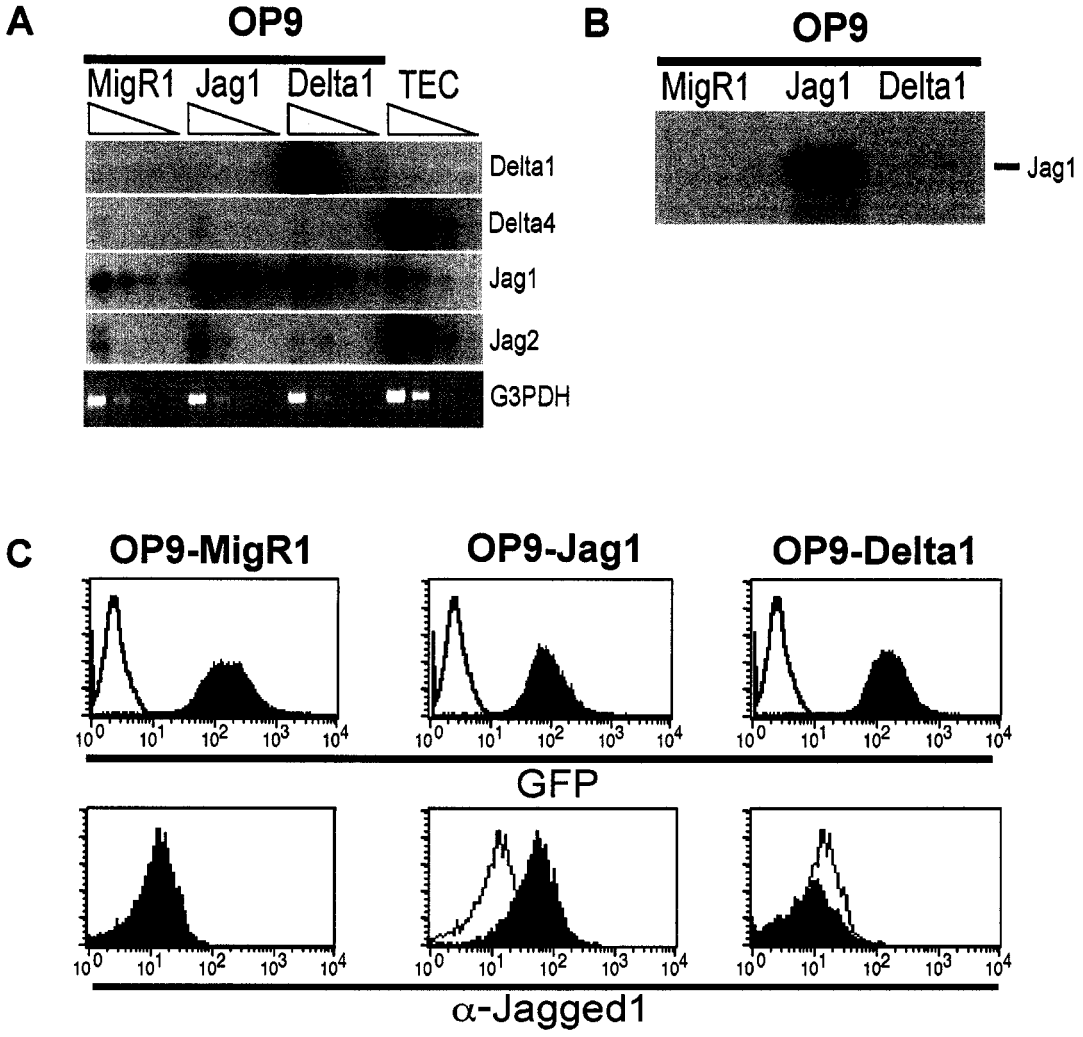


Figure 6: Characterization of OP9 stromal cells expressing Jagged1.

(A) Expression of Notch ligand mRNAs in OP9 stromal cells and normal thymic epithelial cells (TEC) analyzed by RT-PCR followed by Southern Blot. cDNAs were normalized by TaqMan PCR for HPRT, and 5-fold serial dilutions of normalized cDNAs were subjected to PCR for different Notch ligands or G3PDH as a loading control. (B) OP9 cell lysates were immunoprecipitated with an antibody directed against the extracellular domain of Jagged1, and Western Blots were probed with antisera directed against the intracellular domain of Jagged1. (C) Flow cytometric analysis of OP9-MigR1, OP9-Jagged1 (Jag1) and OP9-Delta1 for GFP expression (top panels) and for cell surface expression of Jagged1 (bottom panels). Empty histograms show negative controls. For GFP, control is fluorescence in non-transduced OP9 cells (top panels). For anti-Jagged1 staining, control is OP9-MigR1 cells stained with anti-Jagged1 (bottom panels).

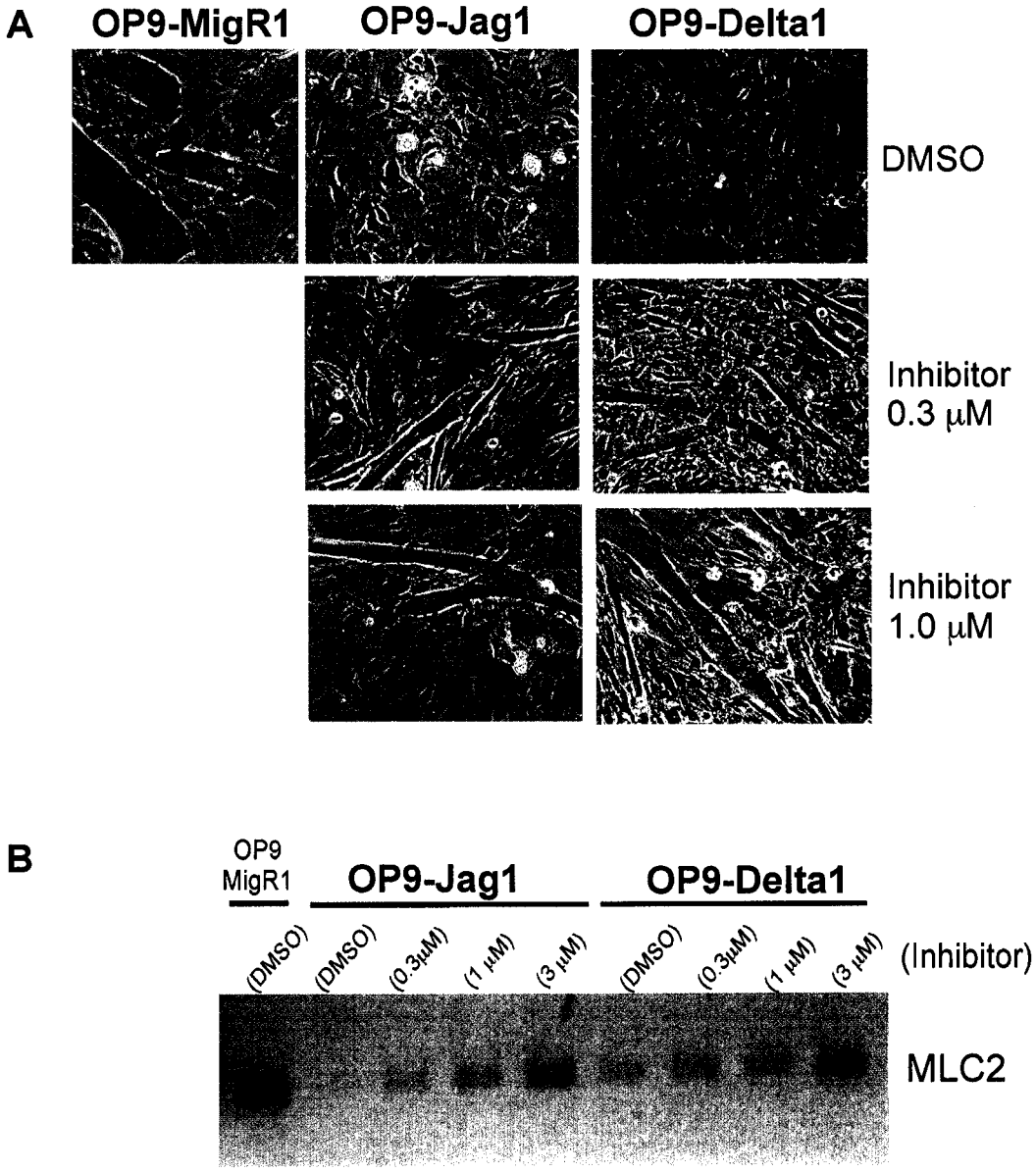


Figure 7: OP9 expressing Delta1 or Jagged1 transmit equivalent Notch signals to C2C12 myoblasts.

(A) Differentiation of C2C12 myoblast cells after 7 days co-culture on OP9-MigR1, OP9-Jagged1 or OP9-Delta1 in the presence of carrier alone (0.1% DMSO) or increasing concentrations of γ-secretase inhibitor X . (B) RT-PCR analysis for expression of a myotube differentiation marker, MLC2, in C2C12 myoblasts shown in (B). Total RNA extracted from a single well was reverse transcribed, and normalized by TaqMan PCR for HPRT.

delivered to C2C12 using a γ-secretase inhibitor L-685,458 (inhibitor X)[74]. The ability of OP9-Jagged1 *vs.* OP9-Delta1 to transmit Notch signals to C2C12 was assessed by examining myotube formation (Figure 7A), or more quantitatively, by monitoring the expression of MLC2 by RT-PCR analysis (Figure 7B). Addition of increasing doses of the γ-secretase inhibitor promoted C2C12 differentiation to similar degrees in OP9-Jagged1 and OP9-Delta1 co-cultures, suggesting that Jagged1 and Delta1 transmit similar levels of Notch signals in C2C12 myoblasts.

Jagged signals promote NK cell development from DN1 thymocytes.

To determine whether Notch ligands differ in their ability to promote specific stages of T cell development, we examined two different precursor populations: bone marrow-derived stem cells (BM-HSC) and the earliest thymic progenitors, DN1 thymocytes (thymus DN1). BM-HSCs were isolated by gating out lineage marker positive cells, and sorting for c-Kit^{hi}/Sca1^{hi} stem cells, and DN1 thymocytes were sorted as lineage negative CD44^{+}/CD25^{-} (Figure 8A). Sorted precursor populations were cultured on OP9-MigR1, OP9-Jagged1 and OP9-Delta1 and their ability to respond to Notch ligands was monitored by examining proliferation, inhibition of B lineage commitment and T cell maturation through the DN to DP stages.

BM-HSCs proliferated well irrespective of whether they received Notch signals (Figure 8B), although the cell type accumulating in these cultures differed dramatically (see below). In contrast, DN1 thymocytes proliferated preferentially in response to Delta1 and expanded poorly on OP9-Jagged1. When cultured on OP9-MigR1, these cells proliferated with delayed kinetics. It appeared from microscopic evaluation of cultures that only individual clones of DN1 thymocytes had the capacity to proliferate in OP9-MigR1 cultures. Limiting dilution analysis confirmed that less than 1 in 300 DN1 progenitors had the capacity to proliferate on OP9-MigR1 (data not shown and[16,75]).

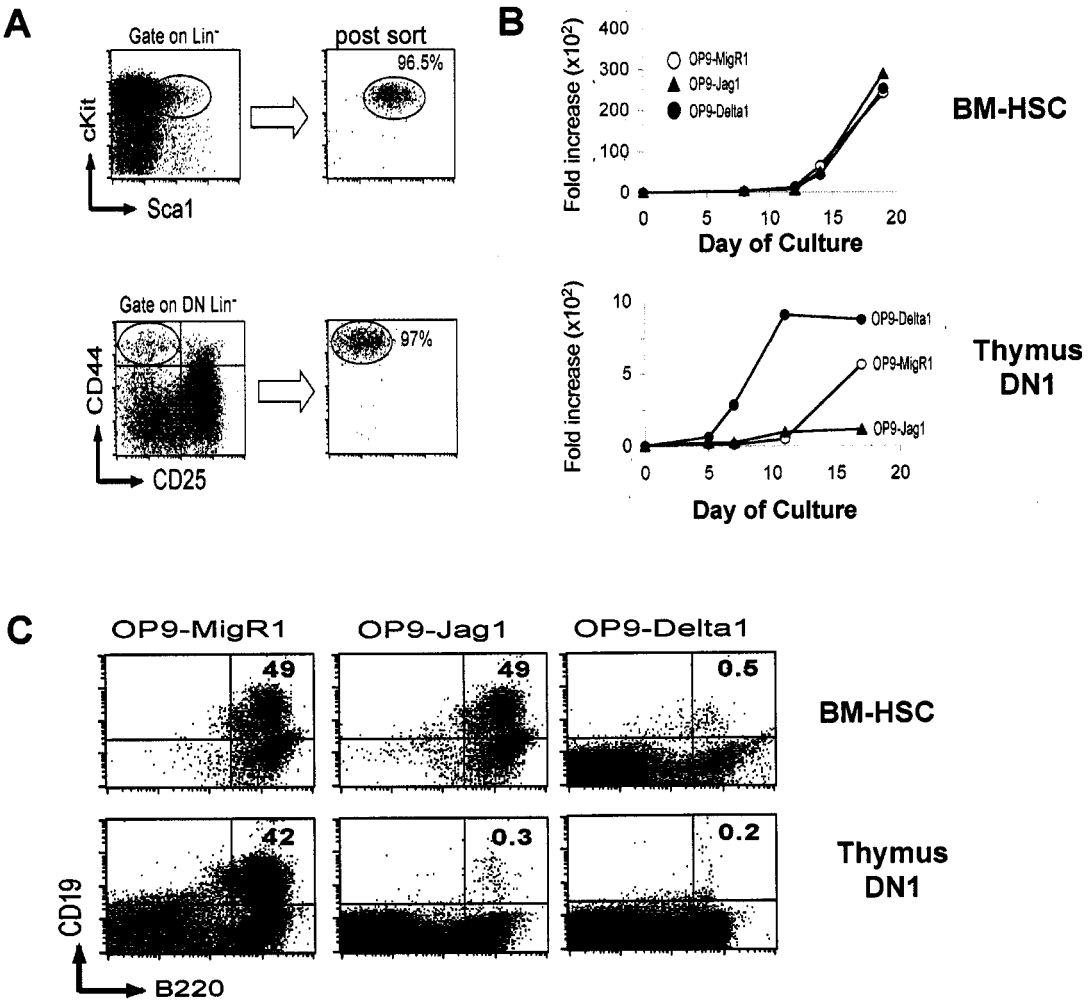


Figure 8: Only Delta1 inhibits B cell development from HSC, whereas both Delta1 and Jagged1 inhibit B cell development in DN1

(A) Isolation of stem cells from bone marrow and thymus by cell sorting. Bone marrow cells were pre-depleted for $B220^+$, $CD11b^+$ and $Gr1^+$ cells, and thymocytes were pre-depleted for $CD4^+$ and $CD8^+$ cells by DYNAL magnetic bead separation. Stem cells were enriched to >96% purity by sorting for Lin^- $cKit^+/Sca1^+$ (BM-HSC) or Lin^- $CD44^+/CD25^-$ (thymus DN1). (B) Proliferation of BM-HSC or thymus DN1 after co-culture on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. Total cell recovery is plotted as fold increase over the initial number of stem cells seeded on day 0. (C) Flow cytometric analysis for B cell markers after 21 days co-culture of BM-HSC, or 11 days co-culture of thymus DN1 on OP9-MigR1, OP9-Jagged1 or OP9-Delta1.

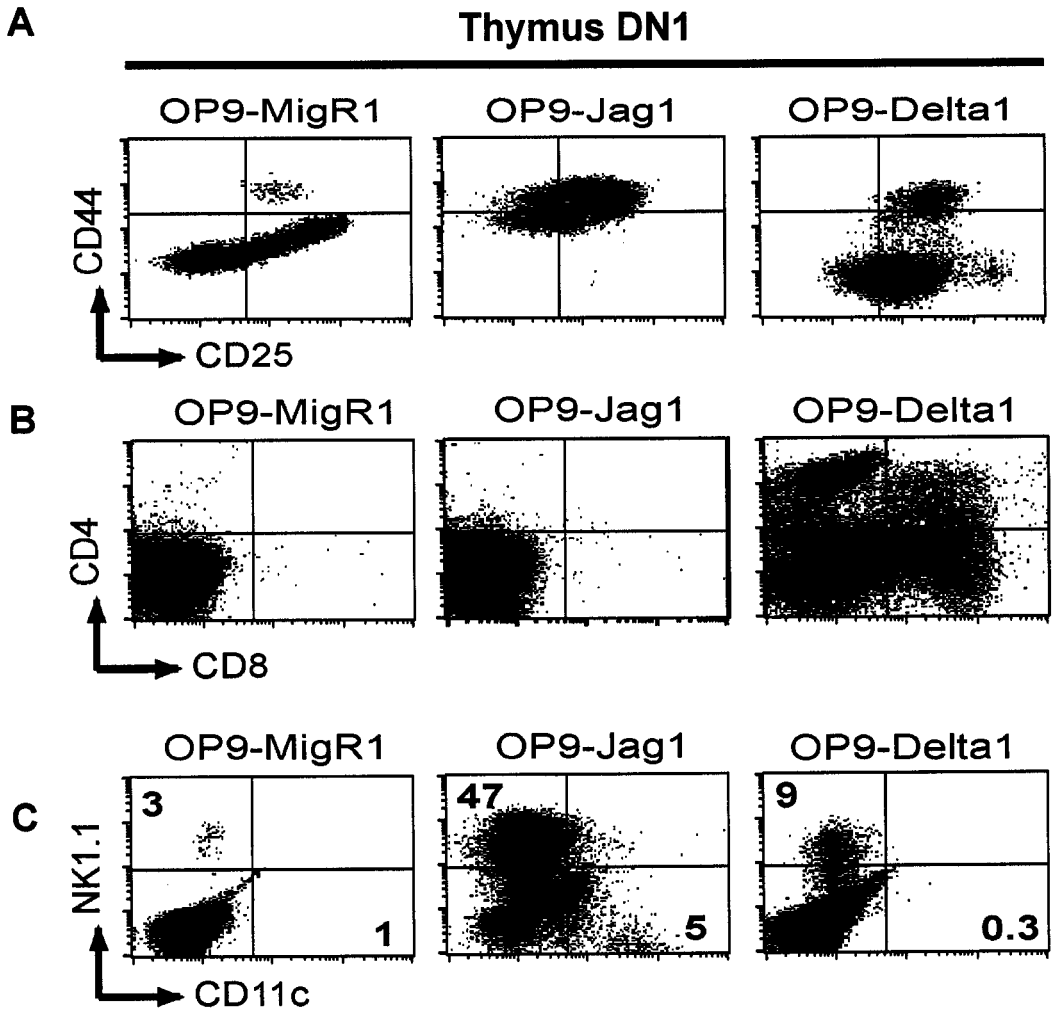


Figure 9: DN1 stimulated with Jagged1 arrest at the DN2 stage, and differentiate preferentially into NK cells

Maturation of DN1 thymocytes into T cells after 26 days co-culture on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. (A) Early DN stages are distinguished by expression of CD44 and CD25, and (B) maturation to the DP stage is monitored by expression of CD4 and CD8. (C) Differentiation of DN1 thymocytes into non-T cell lineages after 26 days co-culture on OP9-MigR1, OP9-Jag1 or OP9-Delta1 by expression of CD11c and NK1.1. Numbers in quadrants refer to the % of gated events in that quadrant.

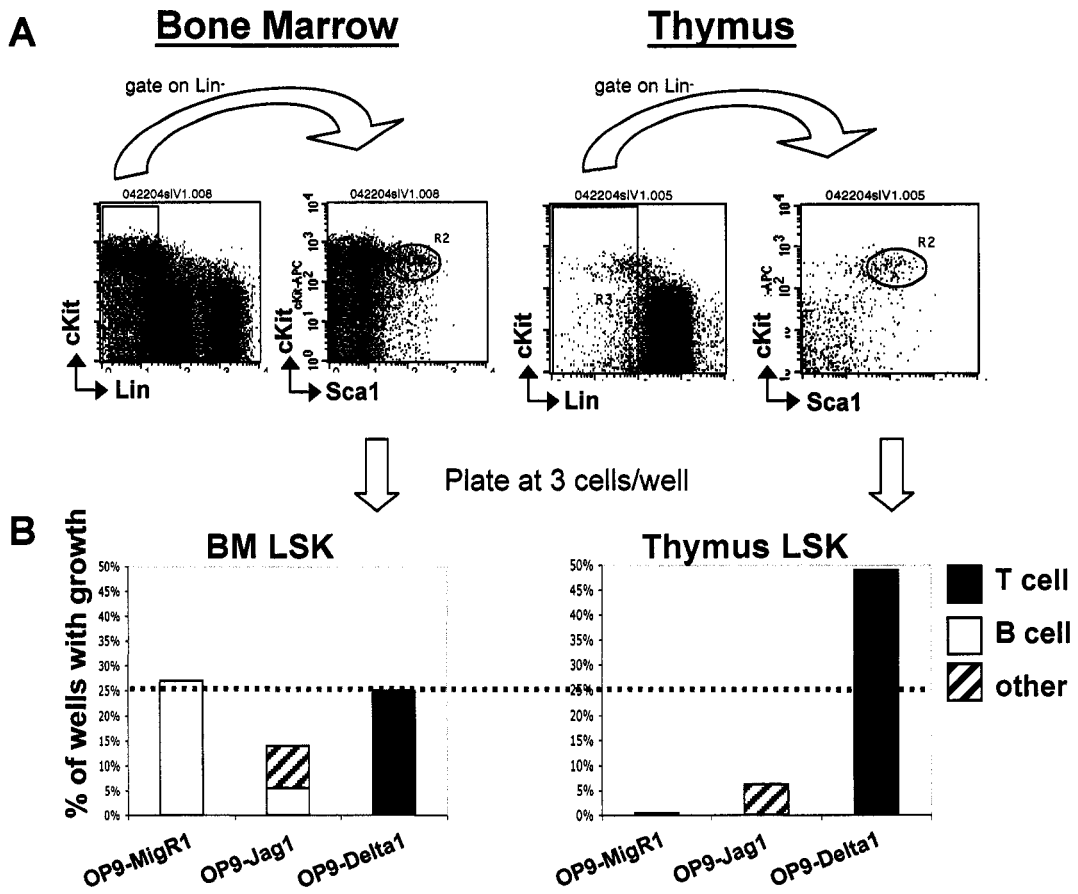


Figure 10: Progenitors from BM or Thymus respond to Jagged signals and differentiate preferentially into NK cells.

Maturation of Lin$^-$ Sca1$^+$/cKit$^+$ (LSK) stem cells isolated from the bone marrow or thymus after 21 days co-culture on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. (A) sort gates for isolation of stem cells. Purified Lin$^-$ Sca1$^+$/cKit$^+$ were plated at 3 cells/well onto monolayers of OP9-MigR1, OP9-Jagged1 or OP9-Delta1. (B) After 21 days wells were scored for growth and phenotyped for expression of Thy1.2 (T cell), CD19 (B cell), B220 or NK1.1(other).

To examine whether co-culture with OP9-Jagged1 and OP9-Delta1 inhibited B cell development in the two cell populations described above, we monitored expression of B220 and CD19 (Figure 8C). As expected from previous data examining human stem cells[67], BM-HSCs developed efficiently into $CD19^+$ B cells when cultured on OP9-MigR1 or OP9-Jagged1, but failed to differentiate into B cells on OP9-Delta1. Instead, BM-HSC cultured on OP9-Delta1 differentiated into $Thy1^+$ T cells (not shown). Similar to BM-HSC, the few DN1 thymocytes that grew, when co-cultured with OP9-MigR1, differentiated efficiently into $CD19^+$ B cells. However, in contrast to BM-HSC, both OP9-Jagged1 and OP9-Delta1 inhibited the differentiation of DN1 thymocytes into $CD19^+$ B cells.

Since Jagged1 signals to thymus DN1 appeared to inhibit B lineage commitment, but failed to induce extensive proliferation of these cells, we examined whether Jagged1 signals were able to promote T cell maturation through the DN to DP stages by monitoring expression of CD44, CD25, CD4 and CD8. As described previously[16], DN1 thymocytes cultured on OP9-Delta1 differentiated efficiently through the DN to DP stages, and by day 26, expressed CD4 and CD8 (Figure 9A and B). These cells expressed intermediate levels of TCRβ characteristic of DP thymocytes (not shown). In contrast, DN1 thymocytes cultured on OP9-Jagged1 did not mature beyond the $CD44^+CD25^+$ stage. Because DN1 progenitors can differentiate into both the NK and dendritic cell lineages, we also examined expression of NK1.1 and CD11c by these cells. In three independent experiments, we found increased percentages (between 20-65%) of $NK1.1^+$ cells in thymus DN1 cells that had been cultured with OP9-Jagged1 (Figure 9C). Because the cells in these cultures did not proliferate well, the absolute number of $NK1.1^+$ cells was not significantly increased in thymus DN1 that had been cultured on OP9-Jagged1 compared to OP9-MigR1 or OP9-Delta1, although we did observe a slight increase of approximately 2-3 fold in some experiments.

Although $NK1.1^+$ cells were gated out in our initial cell sort, it is unclear whether Jagged1 signals were able to promote the differentiation of uncommitted stem cells into the NK cell lineage, or whether Jagged1 allowed the selective

survival of a rare NK1.1$^+$ population by preventing both B and T cell development. To address this question, we sorted ckit$^+$/Sca1$^+$ progenitors from either the bone marrow, or the thymus and plated them at 3 cells/well on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. After 21 days, wells were scored for growth, and phenotyped to differentiate T lineage (Thy1.2$^+$), B lineage (CD19$^+$) or NK lineage (NK1.1$^+$) cells (Figure 10). This analysis revealed that progenitors isolated from the bone marrow or thymus are able to respond to signals from OP9-Jagged1, and the frequency of colonies containing B lineage cells was significantly reduced when bone marrow derived progenitors were cultured on OP9-Jagged1 compared to OP9-MigR1. Whereas, 26% of the wells showed growth of B lineage cells on OP9-MigR1, only 14% of the wells showed growth on OP9-Jagged1, and of these less than half contained B lineage cells. Progenitors derived from either the bone marrow or the thymus were more likely to differentiate into NK1.1$^+$ cells when cultured on OP9-Jagged1.

Jagged1 signals promote γδ T cell development from DN3 thymocytes.

Although Jagged1 signals did not promote the differentiation of DN1 thymocytes past the DN2 stage, Jagged signals may play a role during the later DN3 or DN4 stages of T cell development. To address this, we sorted lineage negative, DN3 (CD44$^-$CD25$^+$) or DN4 (CD44$^-$CD25$^-$) thymocytes and examined their ability to proliferate in response to OP9-MigR1, OP9-Jagged1 or OP9-Delta1. Consistent with previous findings[61,62], DN3 and DN4 thymocytes failed to proliferate on OP9-MigR1, while they proliferated vigorously on OP9-Delta1, demonstrating that they remained dependent on Notch signals in this culture system. DN3 thymocytes proliferated weakly in response to signals from OP9-Jagged1, resulting in at least 10-fold greater cell recoveries compared to OP9-MigR1, but 50-fold lower cell recoveries compared to OP9-Delta1 (Figure 11A). DN4 thymocytes failed to proliferate in response to either OP9-Jagged1 or OP9-MigR1.

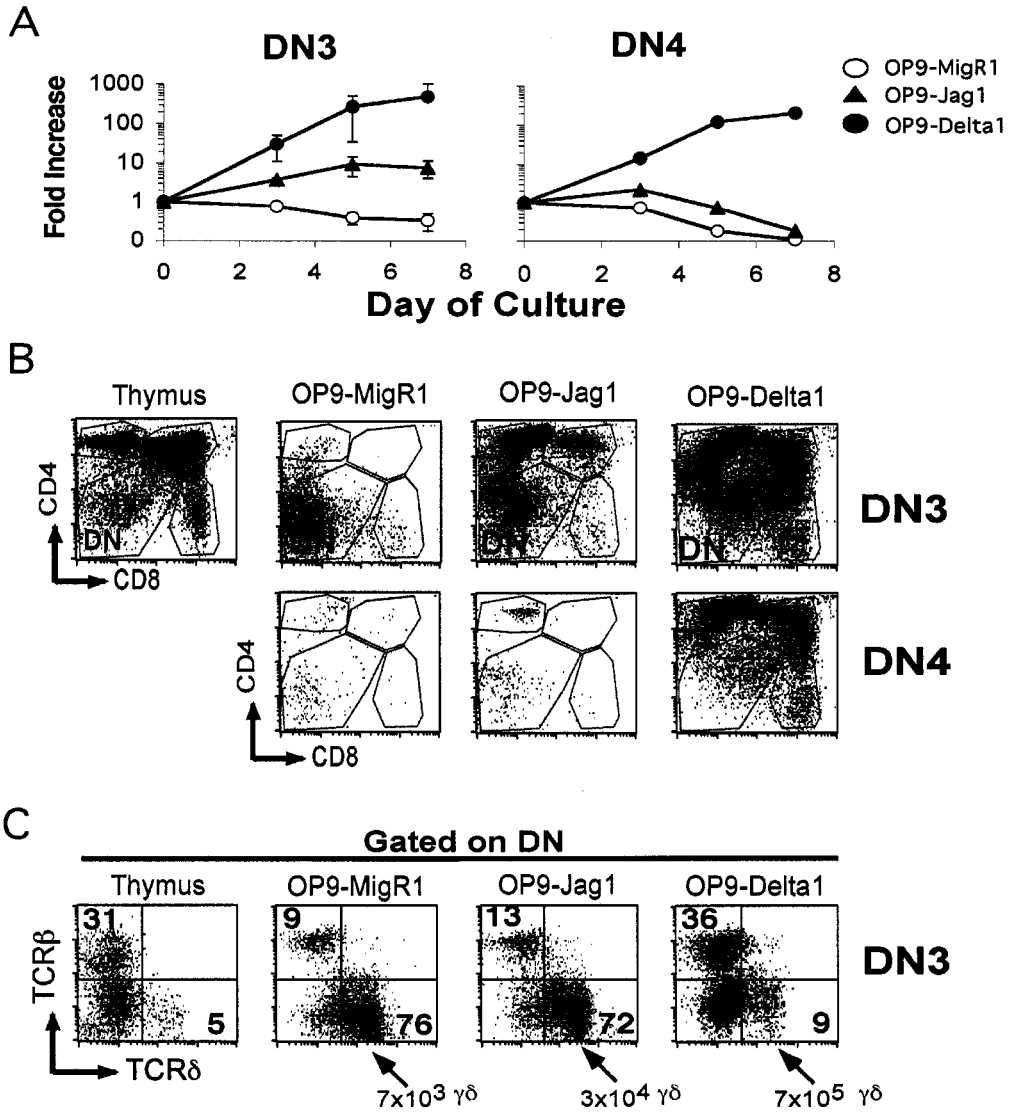


Figure 11: DN3 respond weakly to Jagged and differentiate preferentially to the γδ T cell lineage

Thymocytes were depleted for $CD4^+$ and $CD8^+$ cells by magnetic bead separation and DN3 and DN4 subsets were isolated by sorting for Lin^- $CD44^-CD25^+$ (DN3) or $CD44^-CD25^-$ (DN4) cells to greater than 99% purity. Cells were cultured for 7 days on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. (A) Cell recovery over time in culture. (B) Maturation of DN3 or DN4 thymocytes monitored by expression of CD4 and CD8 surface molecules. Expression of CD4 and CD8 molecules on normal thymocytes is shown as a staining control. (C) Expression of TCRβ and TCRδ in the recovered DN population shown in (B). The total number of γδ T cells recovered per well was averaged from three independent experiments.

To determine whether Jagged1 signals influence the maturation of DN3 thymocytes, we examined expression of CD4, CD8, TCRβ and TCRδ after 7 days co-culture. DN3 thymocytes matured efficiently to the DP stage in response to OP9-Delta1, and appeared to differentiate preferentially into CD4 single positive T cells on OP9-Jagged1 (Figure 11B). These CD4 single positive cells expressed intermediate levels of TCRβ characteristic of immature DP thymocytes (data not shown). Examination of the cells that remained DN after 7 days of culture revealed that DN3 co-cultured on OP9-MigR1 or on OP9-Jagged1 differentiated preferentially into γδ T cells (Figure 11C). Due to the weak proliferation of DN3 in response to OP9-Jagged1, this resulted in a 4-5 fold absolute increase in the total yield of γδ T cells recovered from OP9-Jagged1 cultures compared to OP9-MigR1, but did not improve the yield of γδ T cells when compared to OP9-Delta1. These data demonstrate that DN3 and DN4 thymocytes continue to require Notch signals to proliferate in vitro, and that Jagged1 signals are less potent than Delta1 signals in promoting this proliferation. DN3 thymocytes that are stimulated with no or weak Notch signals, provided by either OP9-MigR1 or OP9-Jagged1, differentiate preferentially into γδ T cells.

The above data demonstrating that Jagged signals are able to prevent B cell development in DN1 thymocytes, but unable to promote T cell maturation; and that Jagged signals are inefficient in promoting the proliferation and maturation of DN3 thymocytes raise an important question. Are Notch signals delivered by Jagged and Delta qualitatively, or quantitatively different? To determine whether Notch signals delivered to immature thymocytes are qualitatively different, we examined the induction of a number of genes that are known to be induced by Notch signaling in T cells. CD25, is induced in thymocytes, as they progress from the DN1 to DN2 stage (Figure 2), and Notch signaling induces CD25 expression in T cells[76,77]. To examine whether Jagged *vs.* Delta signals are able to induce CD25 expression in thymocytes, we sorted DN3 thymocytes and cultured them for 3 days on OP9-MigR1, OP9-Jagged1 or OP9-Delta1, and examined CD25 expression by FACS analysis (Figure 12A).

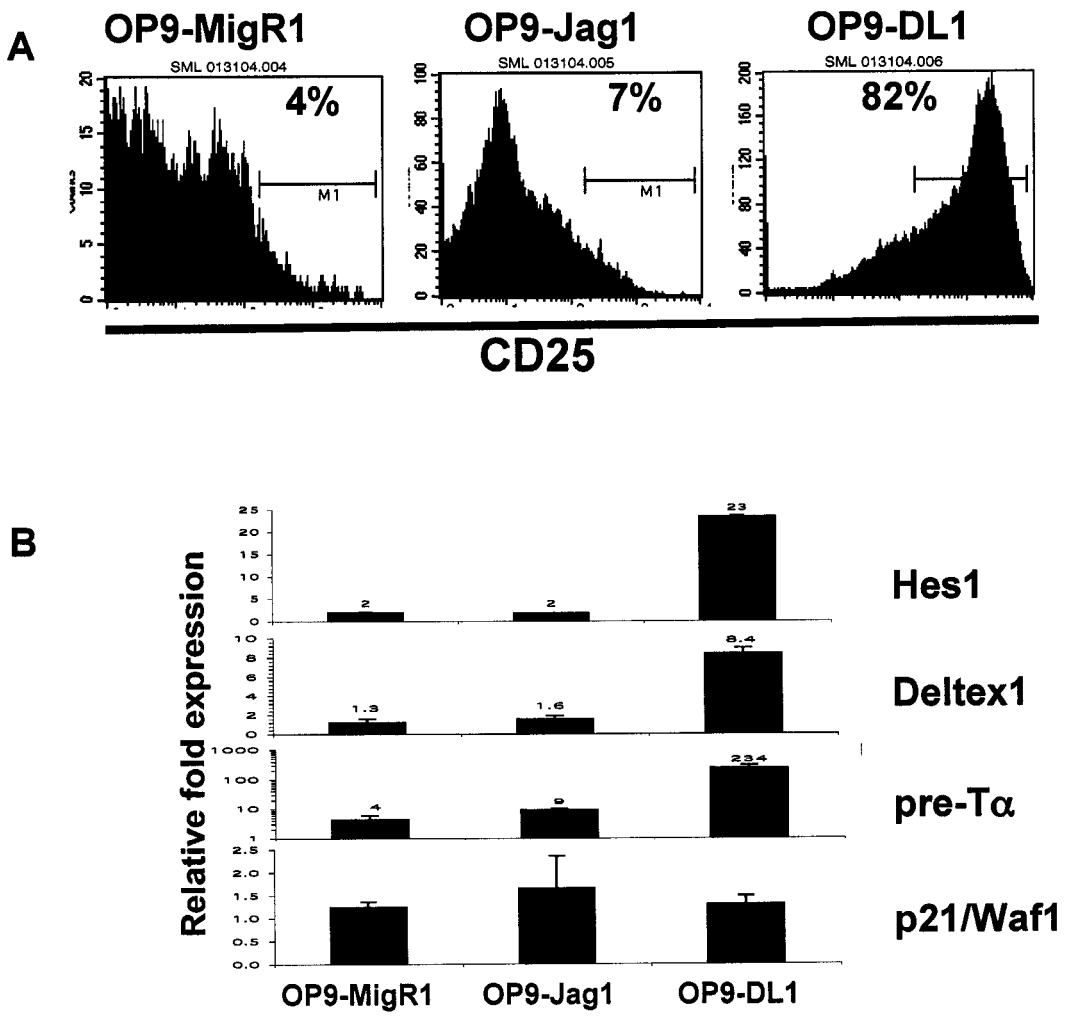


Figure 12: Notch-responsive genes are poorly induced by Jagged1 in thymoctyes and in 1010.

(A) FACS analysis for CD25 expression in DN3 thymocytes cultured for 3 days on OP9-MigR1, OP9-Jagged1 or OP9-Delta. (B) Induction of Notch-responsive genes by TaqMan RT-PCR in 1010 cultured for 24 hours on OP9-MigR1, OP9-Jagged1 or OP9-Delta1.

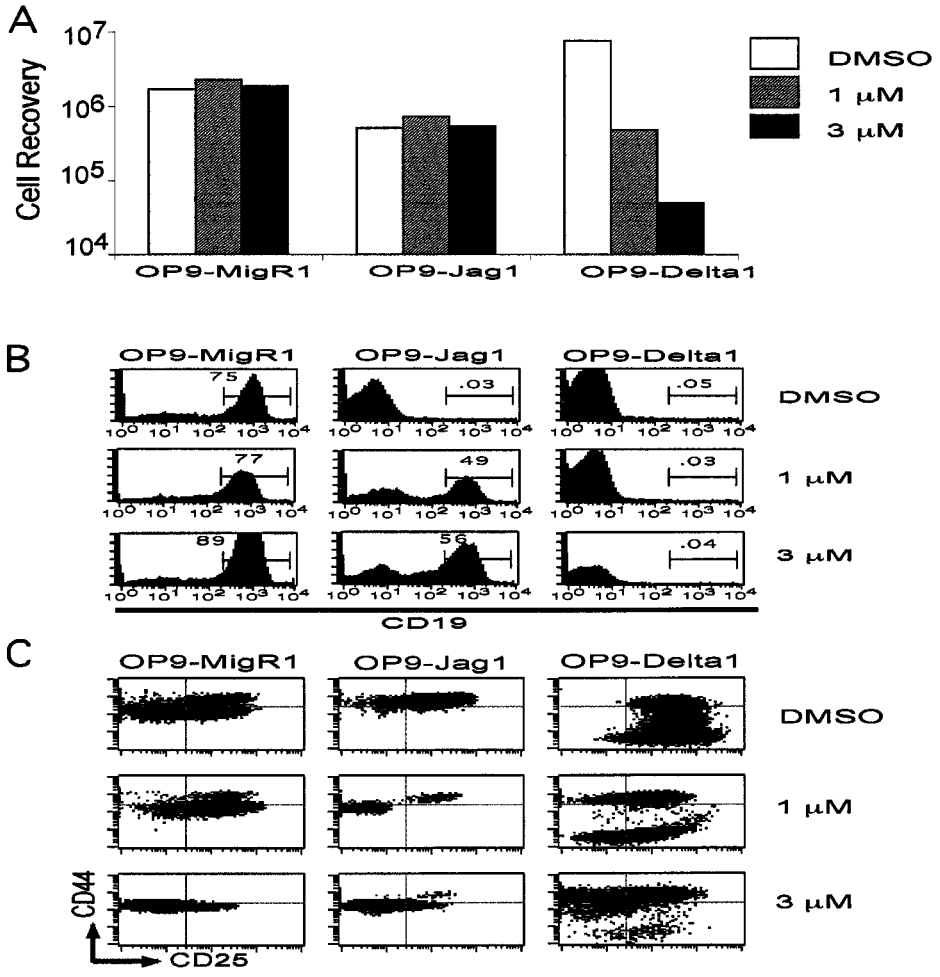


Figure 13: Jagged1 signals are more sensitive to inhibition by a presenilin inhibitor.

DN1 thymocytes were plated at 2000 cells/well on OP9-MigR1, OP9-Jagged1 or OP9-Delta1 in the presence of carrier (0.1% DMSO) or increasing concentrations of inhibitor X. After 12 days co-culture, the cells were counted and examined for expression of differentiation markers. (A) Recovery of DN1 thymocytes per well in the presence of carrier alone (white), 1.0 μM (hatched) or 3.0 μM (black) inhibitor X. (B) Inhibition of B cell development monitored by expression of CD19. (C) Maturation through the DN stages monitored by expression of CD44 and CD25.

Alternatively, we examined expression of 4 known Notch responsive genes, Hes1, Deltex1, pre-Tα[76] and p21/Waf1[37] in a thymoma cell line (1010) after 24 hours co-culture on the above stromal cells (Figure 12B). This analysis revealed that Jagged signals were consistently unable to induce the expression of any of the Notch responsive genes tested.

To compare the intensity of Notch signals delivered from OP9-Jagged1 *vs.* OP9-Delta1 to DN1 thymocytes, we titrated the dose of Notch signals using the γ-secretase inhibitor X. Sorted DN1 thymocytes were cultured on OP9-MigR1, OP9-Jagged1 or OP9-Delta1 in the presence of increasing doses of inhibitor X. After 12 days, the relative intensity of Notch signals was compared by monitoring proliferation (Figure 13A), inhibition of B cell development (Figure 13B) and maturation through the DN stages (Figure 13C). Inhibitor X, at 1-3 μM, did not significantly alter the recovery of DN1 thymocytes cultured on OP9-MigR1 or OP9-Jagged1 stromal cells. In contrast, inhibitor X decreased the recovery of DN1 thymocytes cultured on OP9-Delta1 in a dose dependent manner. DN1 thymocytes cultured on OP9-MigR1 differentiated efficiently into $CD19^+$ B cells, and as expected, this differentiation was not affected by inhibitor X (Figure 13B). As shown previously in Figure 8C, OP9-Jagged1 blocked B cell differentiation, and even the lowest dose of inhibitor X restored B cell development. In contrast, we were unable to completely block signals derived from OP9-Delta1. Even at the higher dose of 3 μM inhibitor X, which blocked proliferation of DN1 thymocytes by >100 fold (Figure 13A), we did not detect any $CD19^+$ B cells. Higher doses of inhibitor X (9 μM) were toxic to the cells. DN1 thymocytes cultured on OP9-Delta1 in the presence of 3 μM inhibitor X appeared to arrest at the $CD44^+CD25^+$ stage (Figure 13C). The above data demonstrate that when DN1 thymocytes are examined, Notch signals transmitted by Jagged1 are less potent than signals tranmitted by Delta1.

Conclusions

The existence of two classes of Notch ligands is highly conserved through evolution[30,39]. Whether this is simply to allow their differential tissue expression, or whether Notch ligands transmit distinct signals is unknown. Our data suggest that in T cell development, Jagged and Delta transmit distinct signals to T cell progenitors and influence the ability of thymocytes to develop along the NK cell or the αβ *vs.* γδ T cell lineages. However, it remains uncertain how Notch signals emanating from Jagged and Delta differ at the molecular level. We envision two likely models. They differ qualitatively, through the activation of distinct downstream events, or quantitatively. Our culture system is not ideally suited to addressing these questions because OP9 stromal cells express a low level of Jagged1 mRNA, that is further induced in OP9-Delta1. Therefore, it is unclear whether our results obtained using OP9-Delta1 reflect the coordinated actions of both Jagged1 and Delta1 signals. Furthermore, we cannot rule out the possibility that expression of Notch ligands in OP9 stromal cells alters the expression of other genes that could influence the ability of the cells to support T cell development.

Our observations that signals from Delta1 cannot be completely blocked by the presenilin inhibitor X, suggest that T cell progenitors do not respond well to Jagged signals and that signals through Jagged *vs.* Delta differ primarily in the overall intensity of Notch signals delivered to T cell progenitors. This model is consistent with a body of data suggesting that Fringe selectively inhibits signals through Jagged, while permitting Notch-Delta signals[41]. The data shown in Figure 13, demonstrating that we were unable to block Delta signals using a presenilin inhibitor, could also suggest that Delta1 is able to activate a presenilin-independent pathway that prevents B cell development. However, this model appears unlikely because it would imply that Delta and Jagged inhibit B cell development through different mechanisms.

We were unable to isolate sufficient quantities of DN1 thymocytes to test directly whether Jagged1 and Delta1 differentially activate Notch responsive

genes. However, we did not see any evidence that Jagged is able to activate a subset of Notch responsive genes in T cells. Most notably, DN1 thymocytes upregulate CD25 and transition to the DN2 stage in response to OP9-Jagged or OP9-Delta, suggesting that CD25 may represent a gene target that could be induced efficiently by both ligands. Although it is not clear whether CD25 is a direct transcriptional target of Notch signals, CD25 is highly induced by Notch signaling in T cells[76,77], and co-culture of DN4 thymocytes on OP9-Delta1 results in potent induction of CD25 expression (data not shown). However, Jagged signals did not maintain CD25 expression in DN3 thymocytes (Figure 12), even though it is clear that DN3 thymocytes can respond to Jagged signals at least partially (Figure 11).

Our observation that stromal cells expressing Jagged alone favor different differentiative fates from DN1 *vs.* DN3 progenitors provides an important clue regarding how Notch signals regulate cell lineage choices during thymocyte development. Our data suggest that Jagged signals do not actively induce specific cell lineage choices, but favor the selection of a default fate (Figure 14). DN1 thymocytes have the potential to differentiate into T cells, B cells and NK cells. In the absence of Notch signals, a subset of progenitors found within the DN1 population differentiate into rapidly proliferating B lineage cells, and potent Delta signals promote T cell development. Jagged signals prevent B cell development, but fail to promote T cell maturation. As a result only NK cells are able to survive. This model is consistent with recent data examining the differentiative potential of DN2 thymocytes demonstrating that DN2 thymocytes differentiate into NK cells in the absence of Notch signals[75].

In the case of committed T cell precursors, we observed that Jagged signals can influence the differentiation of DN3 thymocytes into the $\alpha\beta$ *vs.* $\gamma\delta$ T cell lineages. In this case, our data also suggest that Jagged signals promote $\gamma\delta$ T cell development by promoting a default fate. DN3 thymocytes, which are committed to the T cell lineage, require Notch signals from Delta to proliferate in vitro, even after they have received a productive pre-TCR signal[62]. DN3 thymocytes differentiate preferentially into $\gamma\delta$ T cells when cultured on either

OP9-MigR1 or on OP9-Jagged1 (Figure 11C). However, DN1 thymocytes do not differentiate preferentially into the γδ T cell lineage in response to Jagged signals (data not shown). Together these data suggest that Jagged signals favor γδ T cell development by providing an essential survival signal to DN3 thymocytes, whereas only Delta signals promote the massive proliferation of β-selected DN3 and DN4 thymocytes.

The notion that low doses of Notch signals delivered through the Jagged class of Notch ligands can influence γδ T cell development is supported by evidence obtained in three independent knockout studies. First, T cell progenitors derived from Notch1$^{+/-}$ mice differentiate preferentially into the γδ T cell lineage[64]. Second, inhibition of Notch signals during the DN2/DN3 stage through targeted deletion of CBF1/RBPJκ results in increased development of γδ T cells[65]. And third, Jagged2 signals appear to be necessary for generating normal numbers of γδ T cells, as Jagged2$^{-/-}$ mice produce reduced numbers of γδ T cells in the fetal thymus[78].

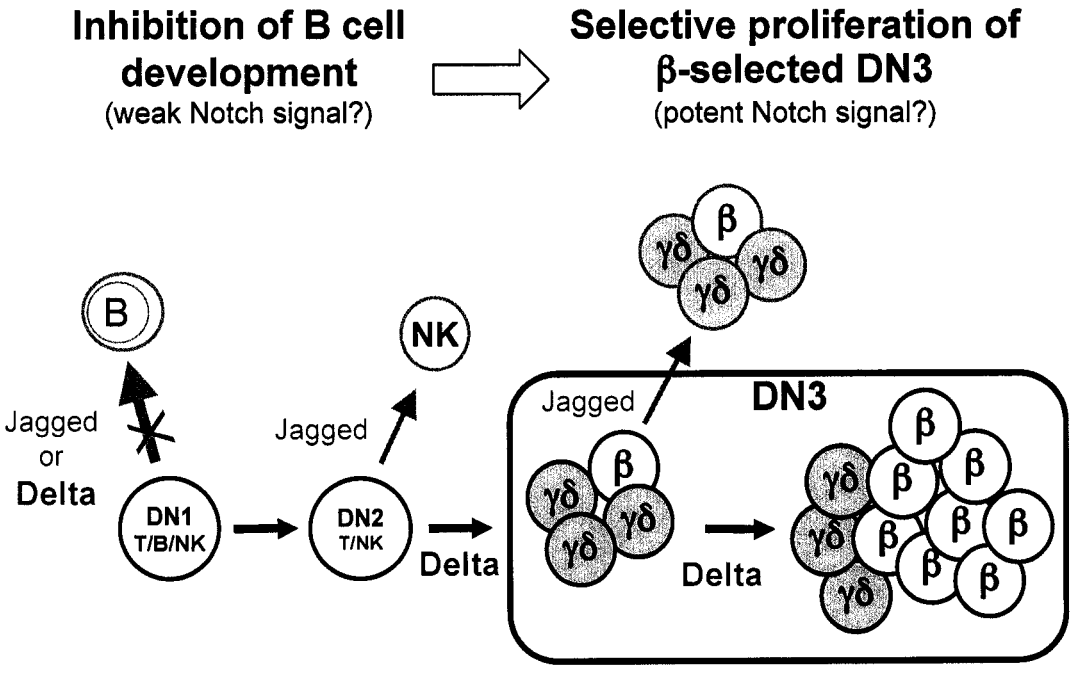


Figure 14: Notch signals promote T cell development in 2 stages.

Jagged signals inhibit B cell development, but do not promote the expansion of DN thymocytes. Jagged signals appear to promote a default cell fate by providing an incomplete Notch signal to T cell precursors. During the DN1 stage, Jagged signals inhibit B cell development, but fail to promote maturation beyond the DN2 stage, and DN1 thymocytes are diverted into the NK cell lineage. During the DN3 stage, Jagged permits some expansion of DN3 thymocytes, but does not promote the proliferative expansion of β-selected DN4 thymocytes, and γδ T cells development is favored.

Chapter III: Targeted deletion of Deltex1.

Notch signals have been proposed to influence the development of T cells throughout their ontogeny, from the generation or maintenance of hematopoietic stem cells[79-81] to their differentiation into mature effector T cell subsets[82]. The above data examining the role of Notch ligands in thymocyte development suggests that subtle differences in the quality or quantity of Notch signals delivered to T cell progenitors can influence the proportions of different cell lineages that develop. However, Notch signals are also regulated by a number of intracellular regulators that influence the transmission of Notch signals within the Notch signal receiving cell[40]. In an attempt to better understand how Notch signals regulate T cell differentiation, our lab has identified a number of genes that are induced by over-expression of Notch-IC in a thymoma cell line. This analysis identified known transcriptional targets of Notch signaling such as Notch1 and Hes1, and several novel genes that had not previously been identified as Notch targets[76] including Deltex1, Meltrin-β, Ifi-204, pre-Tα and Nrarp[83]. We were particularly interested in one of these genes, Deltex1, because Deltex proteins have been clearly implicated in the Notch pathway.

Deltex was first identified in *Drosophila* in a screen for mutations that could suppress the lethal phenotypes resulting from a Notch gain-of-function mutant[84]. Deltex mutants share some of the phenotypic characteristics resulting from mutations in key components of the Notch pathway, and exacerbate Notch loss-of-function phenotypes[85]. Three mammalian homologues to *Drosophila* Deltex have now been identified[86,87] (Deltex1, Deltex2 and Deltex4), which code for a family of cytoplasmic proteins that contain three structural domains (Figure 15A). Domain I contains a WW repeat[88], and has been shown to physically interact with the Notch ankyrin domains[89,90]. Domain II contains a proline rich region that shares homology with SH3-binding domains, and Domain III contains a RING finger domain commonly found in E3 ubiquitin ligases[91].

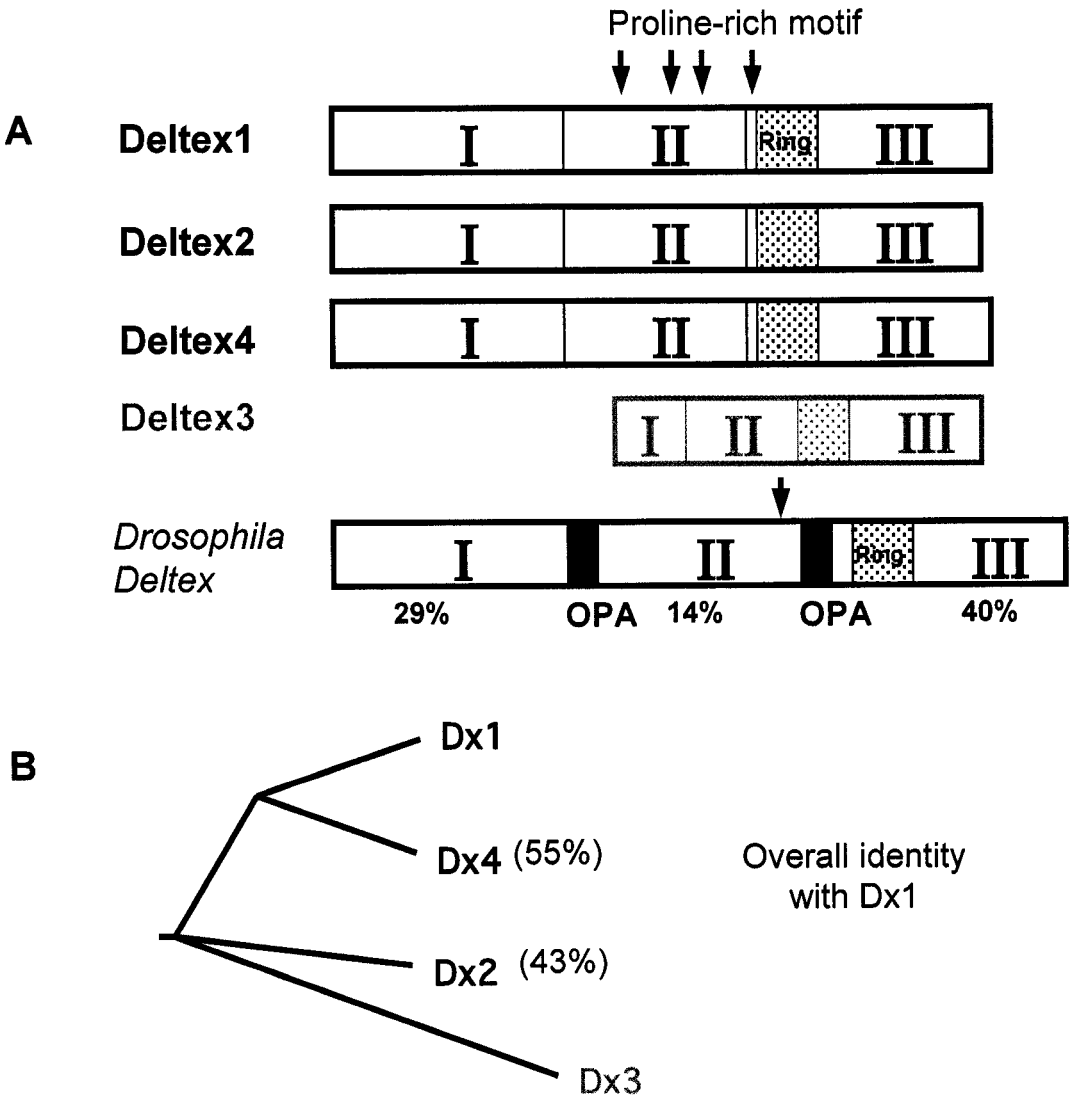


Figure 15: Deltex Family Members

(A) Major structural domains in Deltex family members. Deltex1, Deltex2 and Deltex4 are related to *Drosophila* Deltex, and contain 3 major functional domains. Domain I is required for interaction with the ankyrin domains of Notch-IC. Domain II contains a proline rich region containing a putative SH3 binding sequence. Doman III shows the highest level of similarity between family members, and contains a ring finger domain commonly found in E3 ubiquitin ligases. Numbers under Drosophilla Deltex show similarity of each domain with the indicated domain of murine Deltex1.
(B) Overall homology of murine Deltex family members.

There are several lines of evidence that suggest Deltex plays a crucial role in either regulating or transducing Notch signals, but it is not clear whether Deltex proteins exert a positive or negative influence on the Notch pathway. Studies in *Drosophila*, including the original genetic characterization[84,85] and more recent mutational analysis[90,92], have all suggested that Deltex is a positive regulator of Notch signals. These observations are supported by evidence that, either Notch-IC or Deltex can inhibit transcriptional activity of E-Box containing promoters[93], and either Notch-IC or Deltex1 can inhibit the differentiation of a pro-neural cell line[94]. Nevertheless, other evidence suggests that Deltex1 can inhibit Notch signals. In one study, over-expression of Deltex1 in bone marrow stem cells inhibited T cell development[95], a phenotype similar to that seen in Notch-deficient stem cells. These apparently contradictory results will be resolved by gaining a better understanding of how Deltex and Notch interact at the molecular level in different cellular contexts.

Deltex1 mRNA is induced by Notch signals in T cells

Previous data suggested that Deltex1 mRNA is induced by Notch signals in a thymoma cell line and in transgenic mice expressing Notch-IC in thymocytes[96]. To test whether induction of Deltex1 mRNA is specific to T cells, and whether its induction results from CBF1-dependent Notch signaling, we examined the expression of Deltex1 mRNA in 3T3 fibroblasts or in 2 different thymoma cell lines that had been transduced with retroviruses expressing Notch-IC (Figure 16A). The active intracellular domain of Notch1, Notch3 or Notch1 containing a mutation within the RAM domain, which is required for binding to CBF1 (mM2-2)[33] was expressed in a retroviral vector that also expresses the human CD2 gene, separated by an IRES sequence. Retrovirally transduced cells were purified by panning for the human CD2 marker, and expression of Deltex1 mRNA was examined by Northern blot. This analysis revealed that Deltex1 mRNA is highly induced by either Notch1-IC or Notch3-IC in the thymoma cell lines, but not in 3T3, and that induction of Deltex1 mRNA is dependent on CBF1.

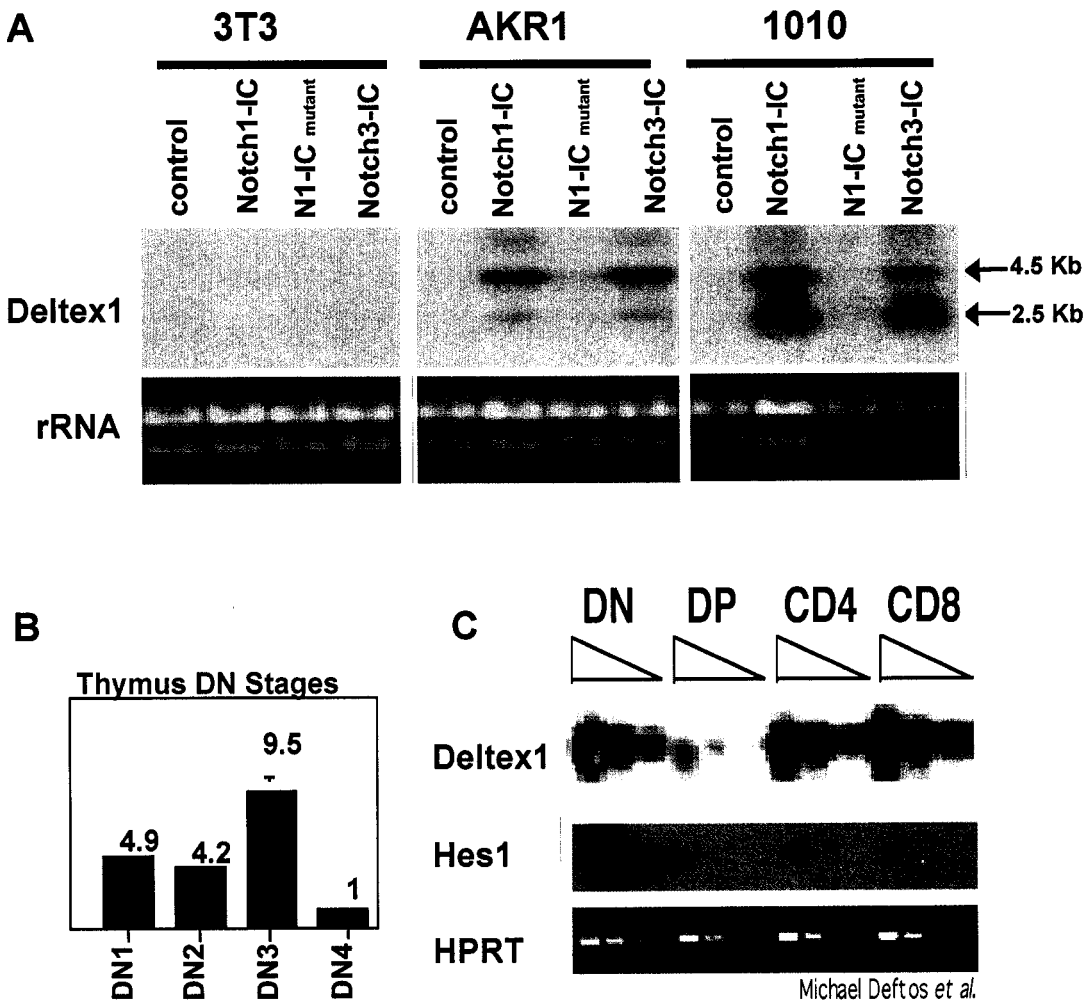


Figure 16: Deltex1 is induced by Notch signals and regulated during T cell development

(A) Northern Blot showing expression of Deltex1 mRNA in cell lines.
Deltex1 mRNA is induced by over-expression the intracellular domain of Notch1 (Notch1-IC) or Notch3 (Notch3-IC) in DP T cell lines AKR1 or 1010, but not in a fibroblast cell line 3T3. Induction of Deltex1 mRNA by Notch1-IC requires the Notch RAM domain. Deltex1 mRNA is not induced by expression of Notch1-mM2-2 ($N1\text{-}IC_{mut}$), which contains a mutation in the RAM domain. (B) TaqMan RT-PCR analysis of Deltex1 expression in sorted DN subsets. cDNA was generated from sorted populations of DN1 (CD44+/CD25-), DN2 (CD44+/CD25+), DN3 (CD44-/CD25+) or DN4 (CD44-/CD25-) thymocytes. (C) RT-PCR analysis of normal thymocyte subsets from Deftos et al. 2000[76].

Notch signaling is regulated during T cell development, and a number of Notch-responsive genes are highly expressed during the DN stages of thymocyte development, down regulated during the DP stage and re-induced as thymocytes mature to into CD4 or CD8 T cells[76]. To test whether Deltex1 is also regulated during thymocytes development, we examined expression of Deltex1 mRNA by TaqMan RT-PCR analysis in sorted DN1, DN2, DN3 and DN4 thymocyte populations. Figure 16B shows that Deltex1 mRNA is most highly expressed during the DN3 stage of T cell development. Expression of Deltex1 mRNA from total DN, DP and SP thymocytes populations from Deftos *et al.*[76] is shown in Figure 16C for comparison.

Over-expression of Deltex1 inhibits Notch signals.

Notch signals are essential for promoting T lineage commitment and bone marrow derived stem cells cannot differentiate into the T cell lineage in the absence of Notch signals[56]. Over-expression of Deltex1 in bone marrow derived stem cell populations has also been shown to inhibit T lineage commitment[49,95], suggesting that Deltex1 acts as a negative regulator of Notch signals in T cells. To confirm this, we tested whether over-expression of Deltex1 could inhibit Notch signals derived from either the natural ligands Jagged1 or Delta1 or from Notch-IC. 1010 thymoma cells expressing Deltex1 were cultured on monolayers of OP9-MigR1, OP9-Jagged1 or OP9-Delta1 (Figure 17A) (see chapter 2 above), or transduced with a retrovirus expressing Notch-IC (Figure 17B), and RNA was isolated after 24 hours. Expression of Hes1 or Deltex1 RNA was measured by TaqMan RT-PCR analysis. This analysis revealed that over-expression of Deltex1 in 1010 effectively blocks the induction of Hes1 in response to Notch signals from OP9-Delta, but does not inhibit Hes1 induced by over-expression of Notch-IC. Retroviral expression of Deltex1 resulted in a 5,000 fold induction of the Deltex1 mRNA, whereas endogenous Deltex1 was induced by approximately 200 fold by over-expression of Notch-IC. Therefore, although it is clear that over-expression of Deltex1 in bone marrow derived stem cells can inhibit T cell

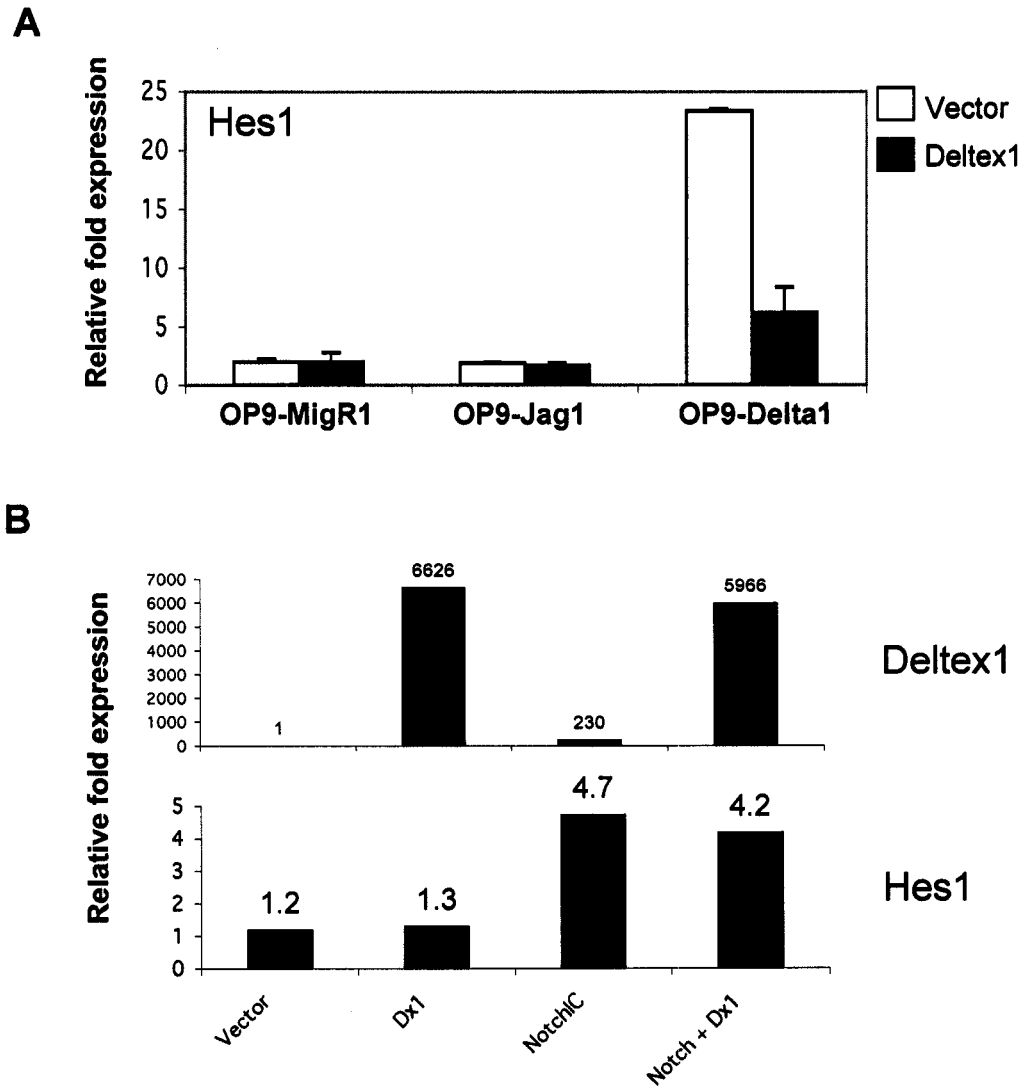


Figure 17: Over-expression of Deltex1 can block Notch signals derived from natural ligands.

(A) Induction of Hes1 mRNA in 1010 or 1010 expressing Deltex1. 1010 cells were transduced with a retrovirus expressing Deltex1 and a human CD5 marker. Deltex1-expressing cells were purified by panning for human CD5, and cultured for 24 hours on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. cDNA was generated from the total RNA preparation, and expression of Hes1 mRNA was monitored by TaqMan RT-PCR analysis. (B) 1010 cells expressing Deltex1 (above) were co-infected with a retrovirus expressing a human CD2 marker and Notch-IC, and human $CD2^+$ cells were purified by panning. cDNA was generated and analyzed for expression of Deltex1 and Hes1 mRNA by TaqMan RT-PCR analysis.

development in vivo, it is not clear from this data, whether the normal levels of Deltex1 that are induced by Notch signaling in thymocytes is sufficient to block Notch signals.

T cell development is normal in Deltex1 knockout mice.

To test whether the normal levels of Deltex1 found in thymocytes influences T cell maturation, we generated Deltex1 knockout (Dx1-KO) mice. The first coding exon of Deltex1 was flanked by LoxP recombinase sequences[97] using standard gene targeting technology in embryonic stem (ES) cells[98] (Figure 18A). Approximately 250 ES clones were screened by Southern Blot analysis, and one clone showing the targeted (2LoxDx1) allele is shown in Figure 18B. The targeted region was removed by transient transfection of the ES cells with Cre recombinase. Chimeric Dx1-KO founders, were bred to C57BL/6 mice and the progeny were interbred to generate homozygous Dx1-KO mice. Northern blot analysis of splenocytes from Dx1-KO mice revealed that the targeted allele produces a truncated mRNA (Figure 18C) that does not code for a functional protein (Figure 18D) as we were not able to detect Deltex1 protein by Western blot analysis using polyclonal antibodies raised against the c-terminal region of Deltex1.

Preliminary analysis of Dx1-KO mice did not reveal any overt developmental abnormalities. We could not detect any defects in the expression of developmental markers on cells isolated from the bone marrow, thymus and spleen. To address the possibility that Dx1-KO cells may exhibit minor developmental abnormalities that we were unable to detect on gross examination, we generated mixed bone marrow chimeras to test whether Dx1-KO bone marrow can compete equally with normal bone marrow. Bone marrow from Dx1-KO (KO) or littermate controls (Wt) that express the allelic marker Ly5.2 was mixed at a 1:1 ratio with bone marrow from wild type mice that express Ly5.1 (competitor), and used to reconstitute lethally irradiated recipient mice. After 3 months, total thymocyte and splenocyte populations were examined

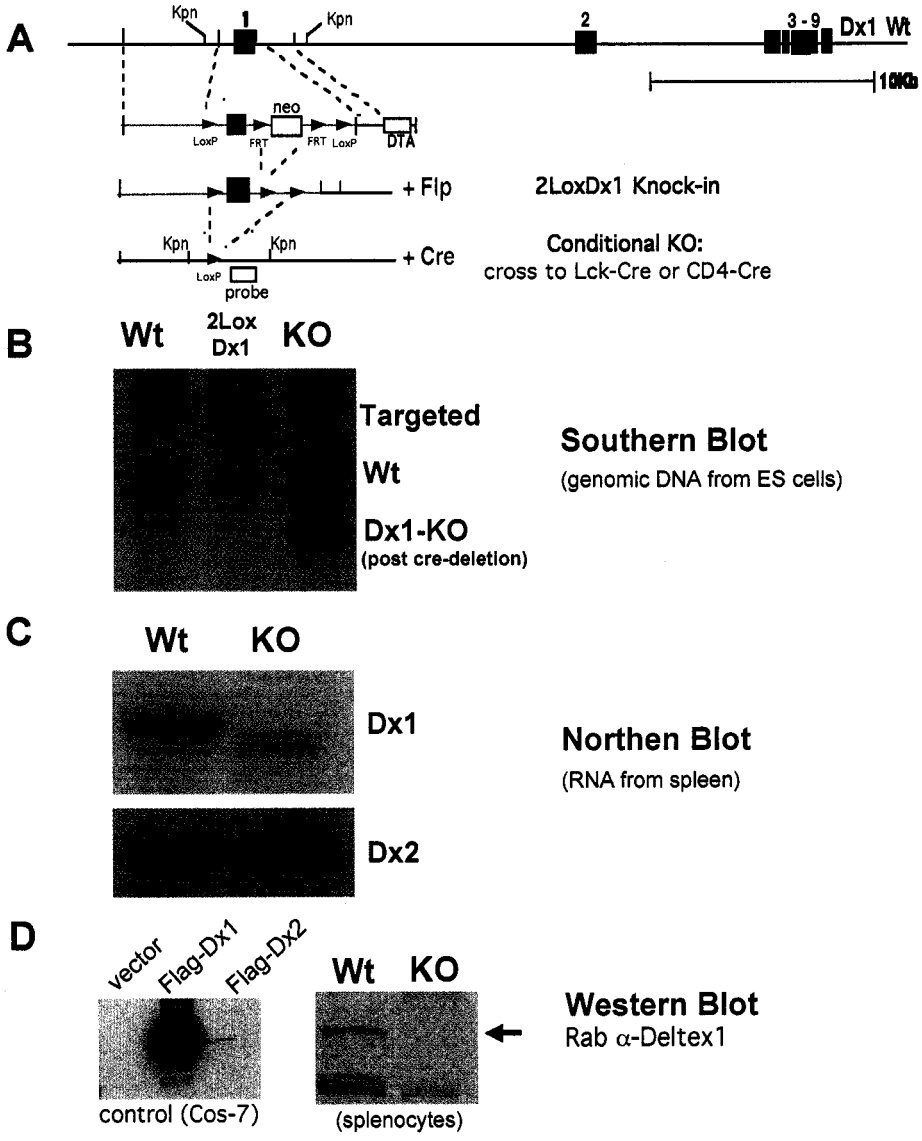


Figure 18: Targeted deletion of Deltex1

(A) Targeting strategy for Deltex1-KO mice. The first coding exon was flanked by LoxP recombinase sites to generate 2LoxDx1 knockin ES cells, and Deltex1 was deleted by transient transfection of Cre recombinase in ES cells. The neo cassette was deleted by breeding Deltex1-KO mice with mice expressing the Flp recombinase under a ubiquitous promoter. (B) Southern blot on genomic DNA isolated from ES cells used to generate 2LoxDx1 knock-in, or Deltex1-KO mice. Genomic DNA was digested with KpnI, and Southern blots were hybridized with the probe indicated in (A). (C) Deltex1 knockout mice produce a truncated mRNA. Northern blot on 10μg of total RNA isolated from spleen of Deltex1 knockout or littermate control mice. Probe is localized within the 3' end of the Deltex1 coding region. (D) Western blot on Cos-7 transfected with Deltex1 or Deltex2 or on splenocytes from Deltex1-KO or littermate control mice. Rabbit polyclonal anti-sera raised against the 3'-end of Deltex1 detects recombinant Deltex1, but not Deltex2 in Cos7 cells.

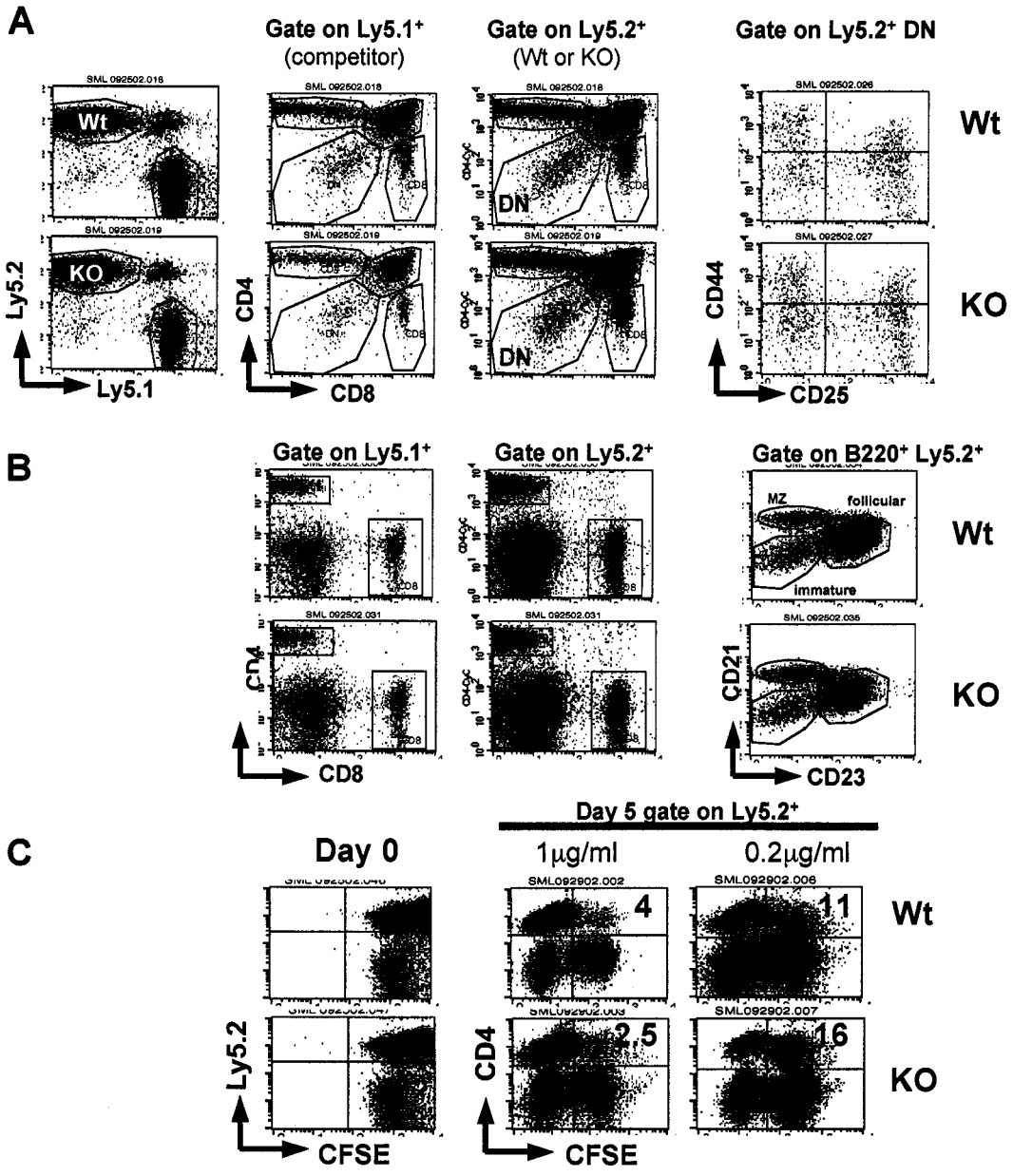


Figure 19: Mixed bone marrow chimeras with Deltex1-KO mice.

(A) Bone marrow form Deltex1-KO or littermate controls (both Ly5.2$^+$) was mixed at a 1:1 ratio with bone marrow from normal (Ly5.1$^+$) mice, and used to reconstitute lethally irradiated recipients. After 3 months, thymus (A) was analyzed for total representation of Ly5.2$^+$ vs. Ly5.1$^+$ cells among CD4$^+$ or CD8$^+$ cells, or for expression of CD44 and CD25 within the CD4$^-$CD8$^-$ (DN) fraction. (B) Total splenocytes were analyzed for normal T or B cell development by expression of CD4, CD8 or B220. Immature, follicular or marginal zone B cells (MZ) were analyzed within the B220$^+$ fraction. (C) In vitro proliferation of CFSE labeled splenocytes from chimeric mice. Total splenocytes were activated with plate bound anti-CD3 for 5 days. Numbers in quadrants represent the total percentage of cells within that quadrant.

by FACS analysis for cell surface molecules. The results from this analysis, shown in Figure 19A reveal that stem cells from Dx1-KO mice differentiate normally into thymocytes and normal ratios of CD4, CD8 and DN populations were represented in the Ly5.2 vs. Ly5.1 fractions. Splenocytes from the above chimeric mice also did not reveal any defects in the differentiation of Dx1-KO stem cells into mature T or B cells based on their expression of CD4, CD8 or B220 (Figure 19B). To test whether Dx1-KO T cells are able to proliferate in response to signals through the TCR, we labeled total splenocytes isolated from the above chimeric mice with carboxy-fluorescein diacetate succinimidyl diester (CFSE) and examined their proliferation in response to plate bound anti-CD3 (Figure 19C). This analysis did not reveal any major defects in the proliferative capacity of Dx1-KO T cells.

In addition to its role in promoting T cell development, Notch signals are necessary for the normal development of marginal zone B cells, a rare subset of splenic B cells which can be distinguished by expression of CD21 and CD23 among $B220^+$ splenocytes[99-101], and there is evidence that Deltex1 is highly expressed in these cells[100]. However, we were not able to detect any defects in the development of MZ B cells in our mixed bone marrow chimeric mice (Figure 19B).

Notch signals can regulate T cell activation

Notch signaling can affect T cell activation and influence the differentiation of mature T cells. However, the mechanism whereby Notch mediates these functions remains uncertain and data published by different groups is somewhat contradictory. The observations (reviewed in[82]) fall into two major categories. A number of groups have proposed that Notch signals can influence the proliferation or survival of T cells after stimulation through the TCR. Others have proposed that Notch signals can promote tolerance *vs.* immunity or influence the differentiation of CD4 T cells into Th1 *vs.* Th2 effector subsets.

A body of evidence suggests that Notch signals play an important role in promoting the survival of mature T cells, potentially through activation of the AKT pathway. This notion arises from two major observations. First, expression of the Notch receptor and its transcriptional target Hes1 are induced in T cells following TCR stimulation. And second, Notch signals appear to be necessary for maximal T cell expansion as T cell proliferation is almost entirely blocked by the addition of a presenilin inhibitor[102] or reduced in T cells that do not express the key Notch signaling component CBF1[65]. A mechanism for how Notch signals affect the survival of T cells through activation of the AKT pathway was proposed in two separate studies, one examining thymocytes, and another examining T cell lines. In thymocytes, Notch signals cooperate with signals through the pre-TCR to promote the expansion of DN3 thymocytes that have undergone β-selection (see Figure 11A). Ciofani *et al* revealed that Notch signals promote the survival of DN3 thymocytes independently of TCR signals, because Notch-dependent survival was also active in thymocytes from $Rag2^{-/-}$ mice. They then proposed that this activity is mediated by a Notch signal-dependent phorphorylation of AKT, as the level of phosphorylated AKT was severely reduced in thymocytes that were deprived of Notch signals, and this Notch-dependent survival signal could be completely replaced by expression of a constitutively active membrane anchored form of AKT[63].

The above data suggesting that Notch signals act through AKT complemented a previous study using a biochemical approach to examine the role of Notch signals in T cell lines[34]. Sade *et al.* proposed that Notch signals promote the survival of T cells by inducing expression of a number of anti-apoptotic genes. They also observed that over-expression of Notch-IC in T cell lines results in increased phosphorylation of AKT. Interestingly, Notch-dependent phosphorylation of AKT appeared to depend on the TCR-associated Src family non-receptror tyrosine kinase $p56^{Lck}$ because Notch signals did not promote survival or phospho-AKT in J.CaM1.6 Jurkat cells which are defective for $p56^{Lck}$.

Finally, third group also noted a link between Notch and AKT. Eager *et al.* used a monoclonal antibody raised against Notch1 that could activate Notch

signaling (as determined by the induction of Hes1 expression) to examine whether co-ligation of Notch1 and the TCR could influence T cell responses. This analysis revealed that both proliferation and the production of cytokines was severely inhibited by costimulation of T cells with anti-Notch1. Although this result is somewhat contradictory to the reports above, which suggested that Notch signals should enhance T cell responses, Eager *et al* reported that Notch signals specifically inhibited the phosphrylation of AKT and GSK-3, while other downstream signaling events such as phosphorylation of ERK were unaffected[103]. Together, the above reports support the notion that Notch signals are somehow integrated with signals through the TCR, and that this integration may be mediated by AKT.

In light of the above data suggesting that Notch signals can influence T cell activation, it is not surprising that Deltex1, a transcriptional target of Notch signaling, has also been proposed to influence T cell activation. However, the mechanism whereby Deltex1 influences T cell responses is less clear, and may not be directly linked to its role in regulating Notch signals. For example, a recent report proposed that Deltex1 mRNA is rapidly down-regulated following stimulation through the TCR, and suggested that Deltex1 can inhibit transcription of the mRNA for IL-2 by targeting MEKK1 for degradation[104]. In this study, over-expression of Deltex1 in T cells inhibited IL-2 secretion, while inhibition of Deltex1 mRNA using RNA interference resulted in increased IL-2 production.

Deltex1-KO T cells show a minor defect in proliferation.

In light of the above evidence that Notch signals and Deltex1 can influence T cell activation, we examined the proliferation of mature T cells from our Deltex1-KO mice. Although Deltex1-KO T cells proliferated normally when we examined CFSE dilution (Figure 19C), we observed a minor defect in the proliferation of Dx1-KO T cells when we monitored thymidine incorporation, particularly when T cells were activated in the absence of exogenous IL-2. Total splenocytes (Figure 20A) or purified T cells (Figure 20B) from Deltex1-KO or

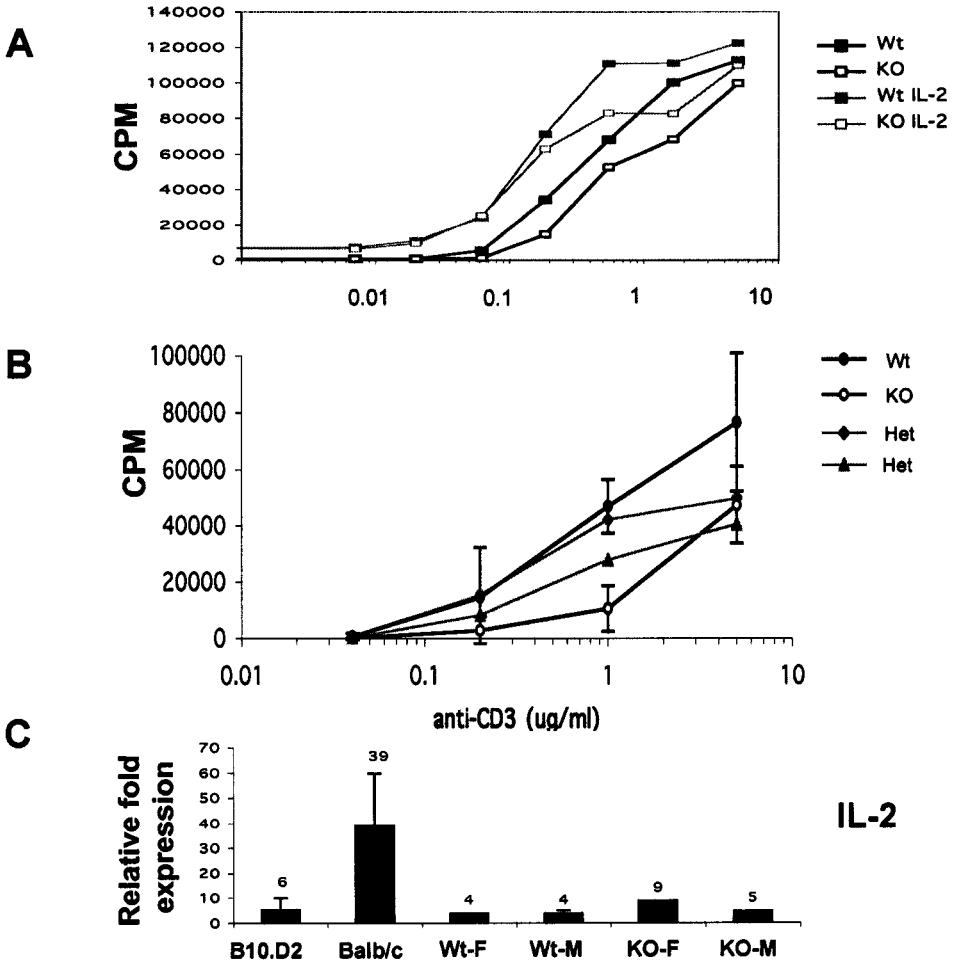


Figure 20: Deltex1-KO T cells show a slight defect in proliferation,

(A) In vitro proliferation of splenocytes from wild type littermate (filled) or Deltex1-KO (open) mice to plate bound anti-CD3 in the absence (black) or presence (gray) of exogenous IL-2. (B) In vitro proliferation of splenocytes or purified T cells (pool of $CD4^+$ and $CD8^+$ T cells purified by positive selection on MACS) from 2LoxDx1-KO X CD4-Cre wild type littermate (black), Deltex1-KO (open) or heterozygous (gray) mice in the absence of IL-2. Each line represents one mouse, error bars show average data from 4 separate assays. (C) Expression of IL-2 mRNA in CD4 T cells isolated from Dx1-KO or littermate control mice by RT-PCR analysis using SYBR green. CD4 T cells were purified from Dx1-KO or littermate controls by positive selection using MACS and stimulated with soluble anti-CD3 and irradiated splenocytes as APCs. After 5 days T cells were restimulated for 6 hours with PMA and ionomycin to generate RNA and cDNA. Each bar represents average data from a pool of 5 female (F) or 5 male (M) mice. cDNAs are from the same experiment shown in Figure 21.

littermate control mice were activated with plate bound anti-CD3 in the presence or absence of exogenous IL-2, and cultures were pulsed with ^{3}H-thymidine after 48 hours. The discrepancy between our results obtained using CFSE dilution *vs.* thymidine incorporation could result if Deltex1-KO T cells enter the cell cycle normally, but do not accumulate to the same degree in culture, for example, if there were a minor defect in their survival. Since Deltex1-KO T cells proliferated at close to normal levels in the presence of exogenous IL-2, we tested whether Deltex1-KO T cells were impaired in their ability to produce IL-2. However, we were not able to detect any significant differences in the IL-2 mRNA levels by RT-PCR analysis (Figure 20C). Our data, suggest that Deltex1-KO T cells are more dependent on exogenous IL-2 than T cells derived from littermate controls, and appears to contradict the results reported by Liu *et al.* proposing that Deltex1 inhibits IL-2 secretion[104].

Notch signals can influence Th1 or Th2 responses.

In addition to its possible role in regulating T cell activation, Notch signals have been proposed by several groups to influence the differentiation of CD4 T cells into either the Th1 or Th2 effector subsets. Several groups have reported somewhat contradictory data suggesting that Notch signals promote the Th2[105] or Th1 cell lineages[106,107]. Amsen *et al.* described that dendritic cells can be induced to express either the Jagged or Delta class of Notch ligands under differing conditions[108]. Delta4 is induced on dendritic cells that have been exposed to a Th1 promoting stimulus LPS (a component of bacterial cell walls), through the conventional Toll-like receptor pathway. In contrast, Jagged1 or Jagged2 is induced on dendritic cells under conditions that have previously been shown to induce Th2 responses. The authors presented evidence that Notch ligands play an important role in inducing polarized T cell responses by showing that antigen presenting cells that have been engineered to express either Delta or Jagged on their cell surface selectively promote the induction of Th1 or Th2 responses respectively. Finally, they proposed a mechanism for how Notch signals are able

to promote Th2 differentiation by showing that the IL-4 gene promoter/enhancer region contains three CBF1/RBPJκ binding sites and that Notch can activate gene transcription via these sites.

The notion that Notch signals play a critical role in promoting Th2 responses is supported by complimentary data that examines CD4 T cell responses in mice in which RBPJκ is deleted specifically in T cells. In separate analyses, Amsen *et al.* and Tanigaki *et al.*[65] both found that the balance of Th1 *vs.* Th2 differentiation is perturbed in these mice. CBF1/RBPJκ deficient T cells differentiate poorly into IL-4 producing Th2 cells, and develop preferentially into IFNγ producing Th1 cells, suggesting that Notch signaling is more important for differentiation into the Th2 lineage.

In somewhat contradictory observations, data from at least two other groups suggest that Notch signaling is important for the differentiation of CD4 T cells into the Th1 lineage. Maekawa *et al.* reported that costimulation of CD4 T cells with a recombinant form of the Notch ligand Delta1 can promote the differentiation of CD4 T cells into the Th1 lineage in an in vitro assay. The notion that activation of Notch signals through Delta1 ligands can promote Th1 responses was supported by in vivo data examining responses to the intracellular pathogen *Leishmania major.* Balb/c mice are normally susceptible to infection with *Leishmania* because they generate an inappropriate Th2 response. However, when these mice were pre-treated with the soluble Delta1 ligand, they were able to generate a protective Th1 response[106]. In another report, Minter *et al.* observed that expression of the Th1 cytokine IFNγ is inhibited when CD4 T cells are activated in the presence of a presenilin inhibitor, whereas expression of the Th2 cytokine IL-4 was not affected by the inhibitor[107]. Minter *et al.* then proposed that Notch signals regulate Th1 responses by activating the promoter for Tbet, a key transcription factor that is essential for Th1 differentiation, and identified CBF1 binding sites within the Tbet promoter that could bind to both CBF1 and Notch-IC in a chromatin immunoprecipitation assay. Together, the above data reveal that Notch signaling can influence peripheral T cell responses

in multiple ways, but also emphasize the fact that how this occurs is by no means clear.

Deltex1-KO CD4 T cells produce elevated levels of Th2 cytokines.

Since Deltex is known regulator of Notch signals, we wanted to examine whether regulation of Notch signals through Deltex1 plays an important role in regulating the outcome of CD4 T cell responses. First, we wanted to determine whether the differentiation of CD4 T cells into the Th1 or Th2 lineages is altered in Deltex1-KO mice. To address this, we examined the differentiation of CD4 T cells into Th1 *vs.* Th2 effectors using an in vitro assay similar to that described by Amsen *et al*[108]. CD4 T cells were isolated from Deltex1-KO or littermate control mice and stimulated with soluble anti-CD3 (0.1μg/ml) and irradiated splenocytes (from the same mouse) as antigen presenting cells under non-polarizing conditions (see legend, Figure 21). After 5 days, T cells were restimulated with PMA and ionomycin for 6 hours and the expression of cytokine RNAs was examined by TaqMan RT-PCR analysis. This analysis (Figure 21A) revealed that Deltex1-KO T cells produce between 2-5 fold higher levels of the mRNAs for the Th2 cytokines IL-4, IL-5 and IL-13 than their littermate controls. Expression of the mRNA for IFNγ was similar between the two groups.

Since T cells isolated from our Deltex1-KO mice appeared to produce elevated levels of Th2 cytokine mRNAs in vitro, we hypothesized that these mice may also develop a skewed Th2 response in vivo. To test this, we infected B10.D2 (resistant strain), Balb/c (susceptible strain), Deltex1-KO or littermate control mice with the intracellular pathogen *Leishmania major* and assessed their ability to control the infection by measuring footpad swelling (Figure 22A) and pathogen load (Figure 22B). We also monitored the expression of Th1 and Th2 cytokine mRNAs in CD4 T cells isolated from the spleen of infected mice 40 days after infection (Figure 22C). This analysis revealed that although CD4 T cells from Deltex1-KO mice produce approximately 2-fold higher levels of Th2 cytokine mRNAs, they were able to effectively clear the *Leishmania* infection.

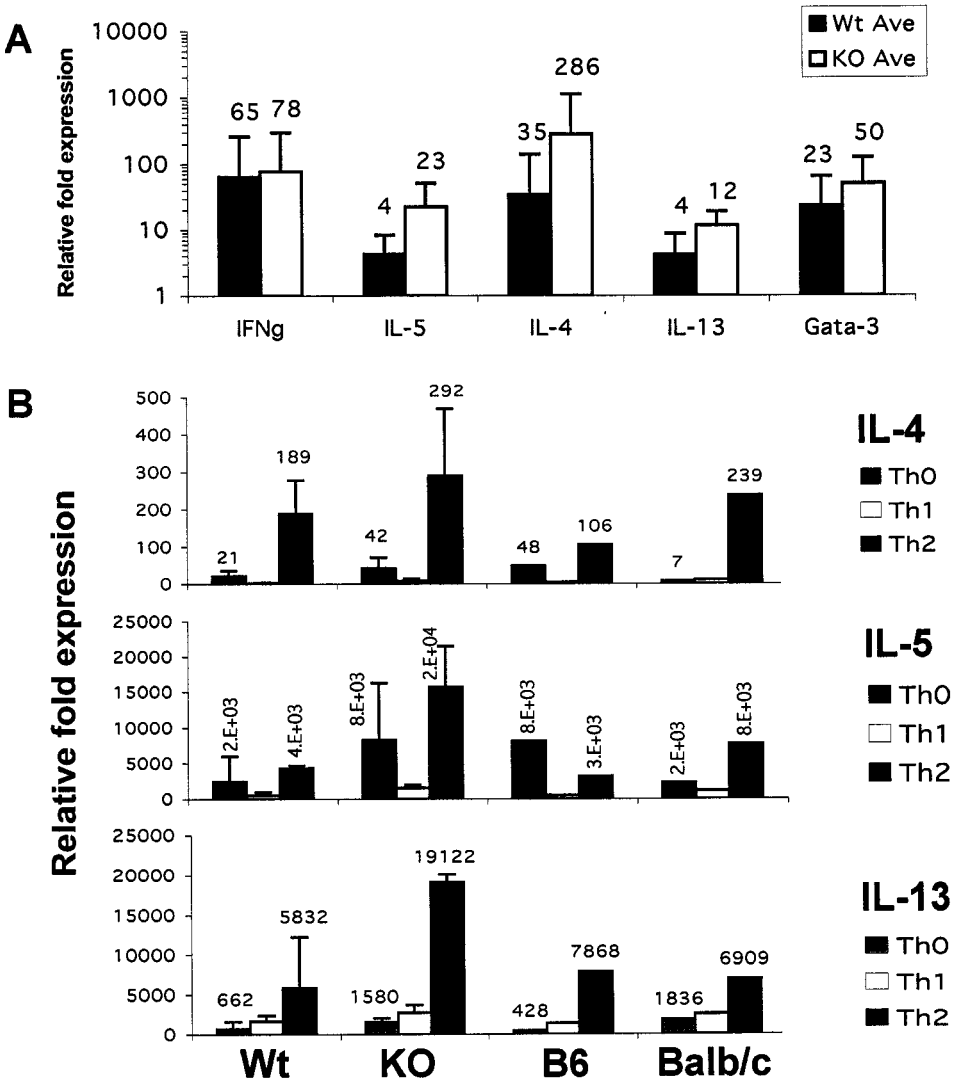


Figure 21: Deltex KO CD4 T cells produce more Th2 cytokines

(A) Expression of cytokine mRNAs in CD4 T cells from Deltex1-KO and littermate control mice. CD4 T cells were isolated from lymph nodes and spleen, and activated with soluble anti-CD3 (0.1ug/ml) and irradiated splenocytes (APC) under non-polarizing conditions (IL-2, anti-IL-4, anti-IFNγ and anti-IL-12). After 5 days, cells were restimulated with PMA + ionomycin for 6 hours to generate RNA. Expression of the indicated mRNAs was detected by TaqMan RT-PCR analysis. Relative fold expression was determined for each experiment by normalizing to expression of β-actin. Error bars show standard deviations from 3 separate experiments using a total of 10 Wt and 9 Deltex1-KO mice. Differences between cytokine expression in Wt vs. KO T cells are reproducible, but not statistically significant by Student's t-test (IL-5 p=.06, IL-4 p=.36, IL-13 p=.10) (B) Elevated Th2 cytokines in Deltex1-KO T cells is retained when primary stimulation is performed under Th2 polarizing conditions. CD4 T cells were activated as in (A) except primary cultures were set up under non-polarizing (colored bars) Th1 (white bars: IL-2, IL-12 and anti-IL4) or Th2 (black bars: IL-2, IL-4 and anti-IL-12) conditions.

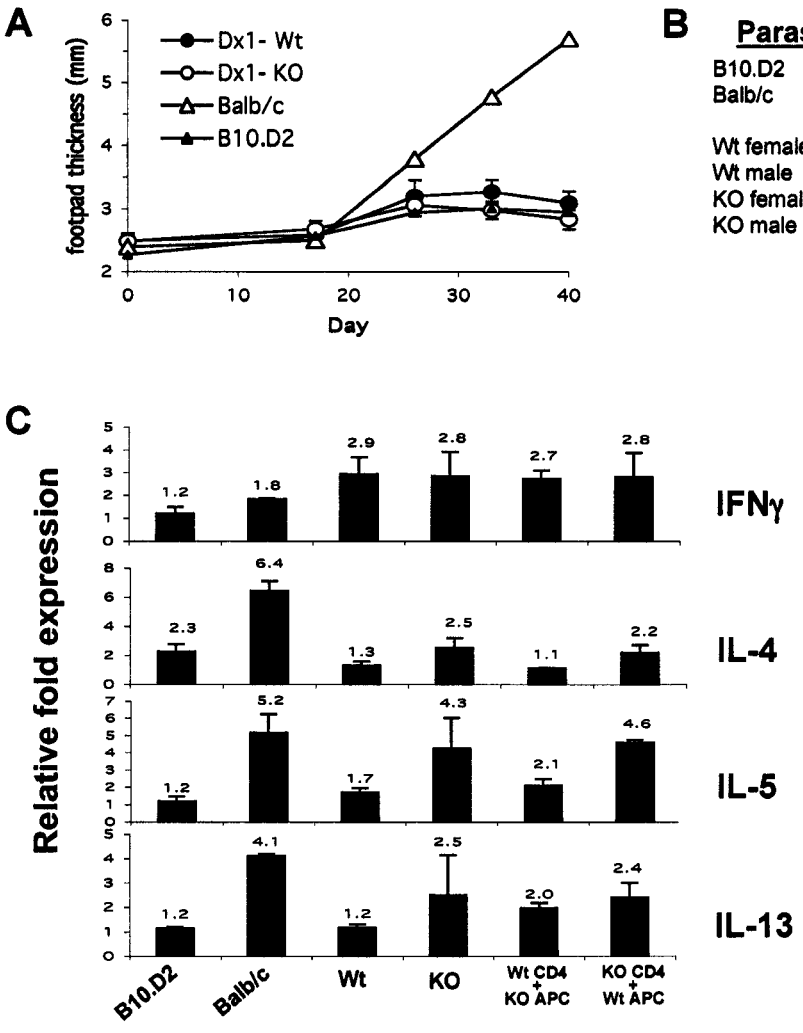


Figure 22: Deltex1-KO mice develop normal responses to infection with *Leishmania major.*

(A) Footpad thickness over time in Deltex1-KO, littermate, B10.D2 or Balb/c mice infected with *Leishmania major*. Error bars represent 3 separate readings at each time point, and 6 mice per group. (B) Parasite burden in footpads from infected mice at day 40. Data is shown as the lowest dilution containing live parasites. (C) Enhanced Th2 cytokine production in Deltex1-KO maps to the T cell rather than the APC. CD4 T cells were isolated from infected mice on day 40, and stimulated with soluble anti-CD3 (0.1μg/ml) and irradiated splenocytes (APC) from Wt or KO mice as indicated (above) under non-polarizing conditions (IL-2, anti-IL4, anti-IL12 and anti-IFNγ). After 5 days, cells were restimulated for 6 hours with PMA and ionomycin to generate RNA and cDNA.

We chose to examine the response of our Deltex1-KO mice to infection with *Leishmania major* because resistance *vs.* susceptibility to *Leishmania* is tightly linked to the ability of recipient mice to generate an effective Th1 response. However, increased production of Th2 cytokines alone is not sufficient to induce disease in resistant strains of mice[109], and our Deltex1-KO mice appear to produce normal levels of IFNγ (Figure 22C). Therefore, it is not surprising that Deltex1-KO mice were able to clear the infection. These data suggest that the elevated Th2 cytokines produced in Deltex1-KO mice may reflect a direct effect on the expression of these cytokine genes, rather that a general skewing of CD4 T cell responses into the Th2 pathway. Supporting this hypothesis, we observed increased levels of Th2 cytokines in Deltex1-KO CD4 T cells that had been activated under Th2 polarizing conditions (Figure 21B), and Deltex1 knockout mice do not produce significantly higher levels of IgG_1 antibodies that are associated with Th2 responses[87].

Notch signals promote T cell leukemia

Notch signals are essential for promoting T lineage commitment, and studies examining the expression of Notch responsive genes in thymocytes revealed that Notch signals are normally activated in T cell progenitors during the early DN stages of their development, but terminated as cells progress to the DP stage[96]. This down-regulation of Notch signals appears to be necessary for normal T cell maturation. When Notch signals are constitutively activated either in bone marrow stem cells or in immature thymocytes during the DN2/DN3 stage, the resulting cells differentiate into an abnormal population of DP T cells that are able to survive outside of the thymus, and eventually develop into a lethal T cell leukemia.

The mechanism whereby constitutive Notch signaling promotes T cell leukemia is not known, however studies examining the effect of Notch signaling in bone marrow stem cells suggest that it is a multi-step process. In studies by Aster *et al.* bone marrow cells were transduced with a retroviral vector

expressing Notch-IC and used to reconstitute lethally irradiated mice. Early examination of the resulting mice revealed an abnormal population of DP T cells within the bone marrow and peripheral lymphoid organs. By 6-15 weeks following bone marrow transplant, clonal sub-populations of these cells (that could be identified by examining the integrated retrovirus by southern blot analysis) began to predominate, presumably because they had undergone neoplastic transformation due to the acquisition of secondary mutations[54,110,111]. Thus, constitutive Notch signaling permits the abnormal differentiation of DP T cells outside of the thymus and facilitates the further differentiation of these cells into leukemic blasts that continue to require Notch signaling to proliferate[32].

Although constitutive activation of Notch signaling in T cell progenitors has been clearly implicated in promoting T cell leukemogenesis, studies examining the role of Notch signaling in other cell types have revealed somewhat contradictory results. For example, Notch signals are important for promoting the differentiation of keratinocytes from proliferating precursor populations. In this case Notch signaling is associated with growth arrest, and Notch signals induce expression of the cell cycle inhibitor p21/Waf1 in these cells[37]. In keratinocytes, Notch signals appear to suppress tumorigenesis and targeted deletion of Notch1 in keratinocytes results in the spontaneous formation of tumors in the skin of affected mice[112]. Together these data reveal that Notch signaling can be pro- or anti-proliferative, and can have opposing effects on the transformation process depending on the cellular context.

Deltex can inhibit or enhance Notch-induced leukemia.

Deltex1 mRNA is highly induced by Notch signals in T cell lines (Figure 16) and DP thymocytes isolated from transgenic Lck-Notch-IC mice[76]. To test whether Notch mediated leukemogenesis is altered in the absence of Deltex1 expression, we over-expressed Notch-IC in bone marrow stem cells from Deltex1-KO or littermate control mice using a retrovirus expressing murine Notch-IC and GFP. The resulting bone marrow was used to reconstitute lethally

irradiated C57BL/6 mice. Bone marrow preparations contained between 10-30% GFP^+ cells 2 days after retroviral transduction. After 22-28 days mice were sacrificed and total cell populations from bone marrow and spleen were counted and analyzed by FACS analysis. Control mice that had received bone marrow expressing GFP alone contained normal ratios T cells and B cells, and the total fraction of GFP^+ cells was unchanged. In contrast, the majority of cells recovered from mice that had received Notch-IC expressing stem cells were GFP^+ and expressed CD4, CD8 and CD25, indicating that the Notch-IC transduced cells had outgrown the non-transduced cells (data not shown).

The total number of cells recovered from the bone marrow and spleen of the above mice is shown in Figure 23A. The spleens recovered from mice that had received Notch-IC transduced bone marrow were highly enlarged, and yielded approximately 4 times more cells than spleens from mice that had received bone marrow expressing MigR1. Spleens isolated from mice that had received Deltex1-KO bone marrow expressing Notch-IC were further enlarged and we recovered 4-fold more cells compared to littermate controls. This analysis suggested that Notch induced leukemia progressed more rapidly in the absence of Deltex1. We did not observe any overt phenotypic differences in leukemic T cells from the two groups of mice, suggesting that the accelerated leukemogenesis observed in Deltex1-KO progenitors resulted from an increase in the number of transformed clones rather than improved growth or survival of the Deltex1-KO cells. To monitor clonal expansion of Notch-IC transduced stem cells, we visualized the integrated retrovirus by Sourthern blot analysis on genomic DNA isolated from either the bone marrow or spleen of leukemic mice (Figure 23B). By this analysis, we were unable to detect distinct bands in genomic DNA isolated from the bone marrow, whereas DNA isolated from the spleen revealed several distinct bands, suggesting that individual clones harboring the integrated retrovirus had expanded within the peripheral lymphoid organs. The frequency of these clones was significantly higher in splenocytes derived from Deltex1-KO leukemias than those derived from wild type littermate controls.

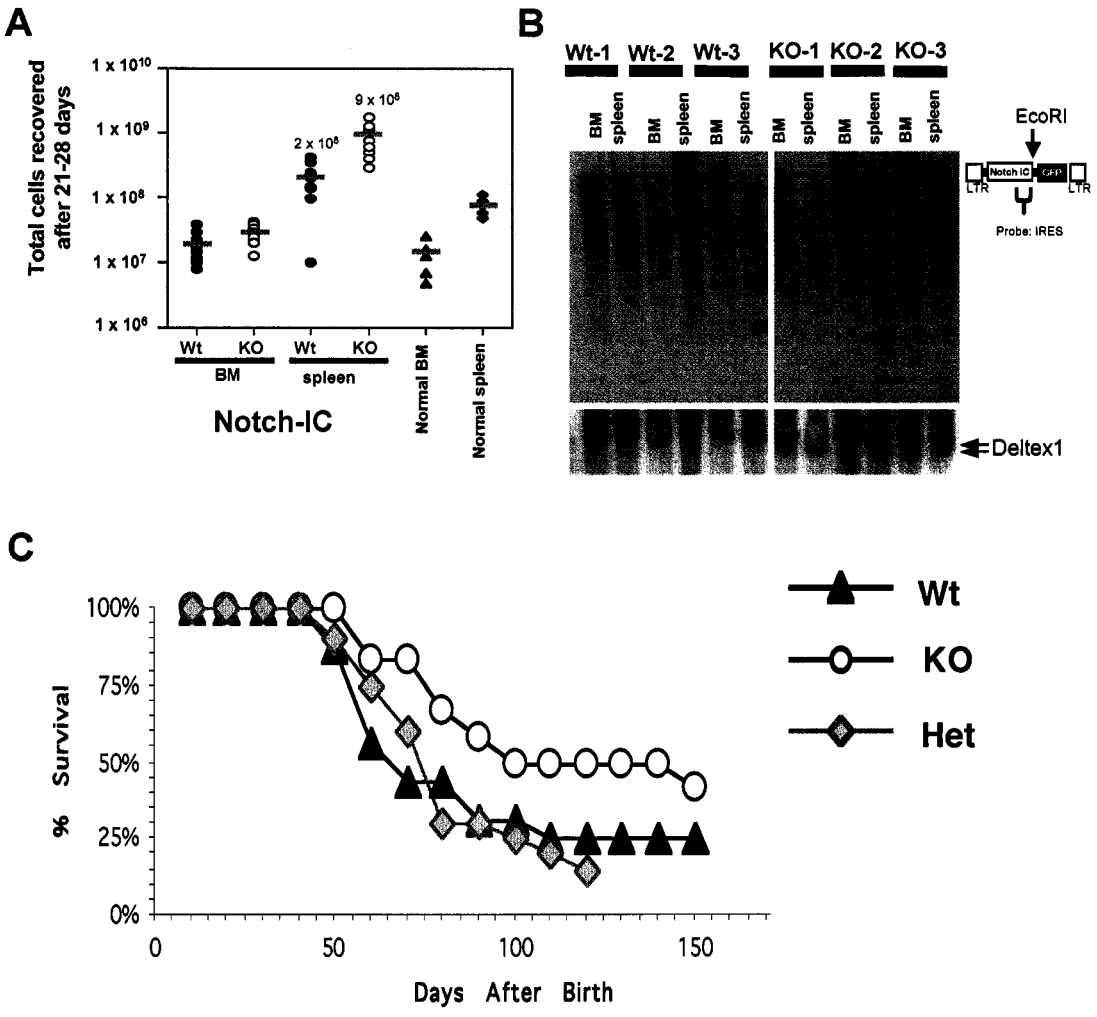


Figure 23: Examination of Notch mediated leukemia in Deltex1 knockout mice.

(A) Bone marrow from Deltex1-KO or littermate control mice was infected with a retrovirus expressing the active intracellular domain of Notch1 (Notch-IC) and used to reconstitute lethally irradiated recipients. 21-28 days after bone marrow transplantation, the bone marrow and spleen of recipient mice was populated with abnormal $CD4^+CD8^+CD25^+$ leukemic T cells (not shown). The total number of cells recovered from bone marrow (BM) or spleen of Notch-IC transduced or empty vector controls is shown. Each point represents 1 mouse, and numbers indicate average data from 2 independent experiments using a total of 9 Wt and 9 Deltex1-KO mice. (B) Southern blot analysis to detect integrated retrovirus in leukemic T cells from the bone marrow or spleen of 3 Wt and 3 KO mice shown in (A). Genomic DNA was digested with EcoRI and probed with either the IRES (upper panels) or with Deltex1 (lower panels). (C) Survival of $Rag2^{+/-}$ X Lck-Notch-IC X $Deltex1^{+/+}$ (Wt), $Deltex1^{+/-}$ (Het) or $Deltex1^{-/-}$ (KO) mice. A total of 16 Wt, 12 KO and 20 Het mice were included in the analysis.

The above analysis reveals that Notch induced leukemia is more severe in the absence of Deltex1. Increased leukemogenesis could result from an increase in the frequency of stem cells that are able to differentiate into T cell precursors, improved proliferation or survival of the cells at some stage during their maturation or an increase in the frequency of secondary mutations leading to oncogenic transformation. To help distinguish between these possibilities, we examined Notch induced leukemogenesis using a different model whereby Notch signals are activated under the control of the Lck-proximal promoter, which is not induced until the DN2 stage of thymocyte maturation. To this end, we crossed our Deltex1-KO mice to transgenic Lck-Notch-IC mice. Examination of a cohort of 16 Lck-Notch-IC/Dx1$^{+/+}$ and 12 Lck-Notch-IC/Dx1$^{-/-}$ mice suggested that Notch induced leukemia is less severe in Deltex1-KO mice when Notch signaling is initiated later, during the DN stages of thymocyte development (Figure 23C). Together, these data indicate that Deltex1 has opposing effects on the severity of Notch induced T cell leukemia depending on the stage of thymocyte maturation during which Notch signaling is first activated.

Chapter IV: Inhibition of Deltex1, Deltex2 and Deltex4 in T cells.

The above analysis reveals that T cell development is mostly normal in mice carrying a targeted deletion in the Deltex1 gene and could indicate that Deltex does not have an important role in regulating Notch signals during T cell development. However, Deltex belongs to a family of at least 3 related molecules (Figure 15). Deltex1 and Deltex2 were both shown to interact with the intracellular domain of Notch using a yeast 2-hybrid assay[86], suggesting that loss of Deltex1 could by compensated by the presence of other Deltex family members. To test this possibility first we wanted to confirm that Deltex1, Deltex2 and Deltex4 are able to inhibit Notch signals. To this end, we examined the ability of the 3 Deltex homologues to block Notch signals using a cell line carrying a stably integrated reporter plasmid in which the CBF1 binding element (8xCBF1) is linked to the expression of GFP (Figure 24A). Over-expression of Notch-IC in these cells acivates CBF1 which can be detected by FACS analysis for GFP expression. We co-transfected myc-tagged Notch-IC with flag-tagged Deltex1, Deltex2 or Deltex4 and analyzed GFP expression after 24 hours. The overall levels of Notch-IC and Deltex family members was monitored by Western blot analysis for the myc or flag tags (Figure 24B). Activation of GFP expression in the presence of the 3 different Deltex proteins was plotted as the percent of GFP^+ cells in cultures co-expressing Deltex and Notch-IC compared to cultures expressing Notch-IC alone (Figure 24C). This analysis reveals that Deltex1, Deltex2 and Deltex4 can inhibit Notch signals equivalently in Cos7 cells.

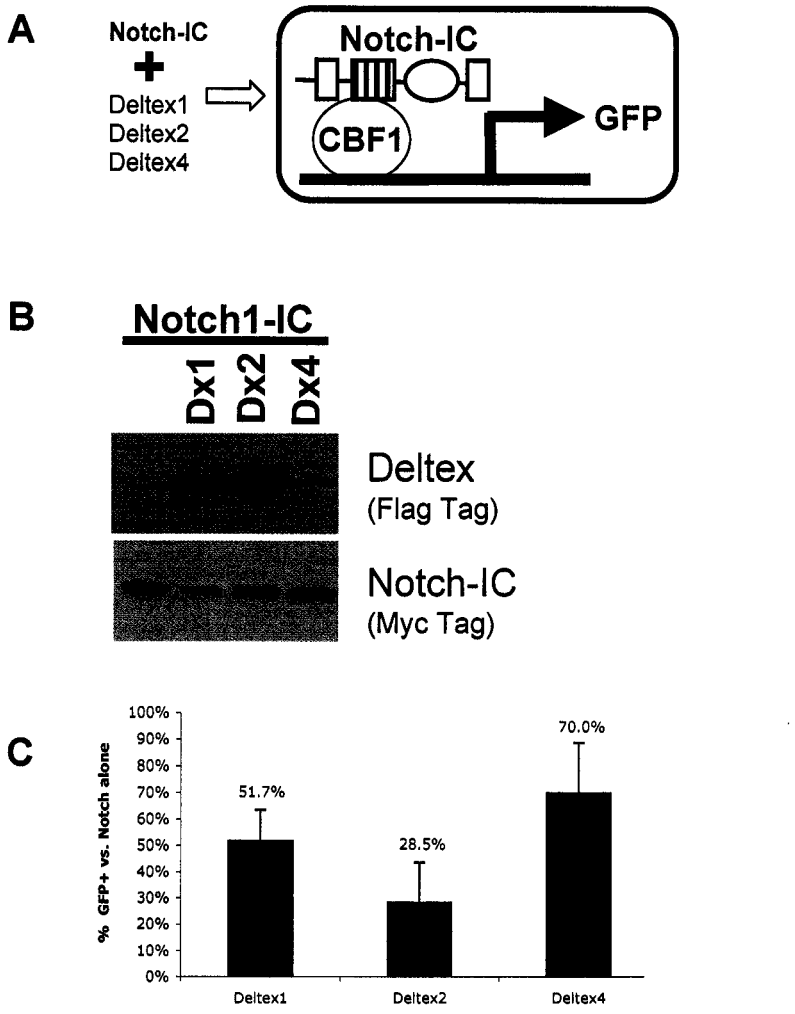


Figure 24: Deltex1, 2 and 4 can inhibit Notch signals

(A) Cos-7 cells expressing a CBF1 responsive element (8x wtCBF1 linked to GFP were transiently transfected with myc-tagged Notch-IC alone or co-transfected with myc-Notch-IC and flag tagged- Deltex1, Deltex2 or Deltex4, and the total % GFP^+ cells was determined by FACS analysis.
(B) W. Blot on cells shown in (C) to compare the level of Notch-IC (anti-myc) or Deltex (anti-flag).
(C) Average inhibition of $\%GFP^+$ in cells cotransfected with Deltex1, Deltex2 or Deltex4 compared to Notch-IC alone. Error bars show average data from 4 independent transfections using Notch1-IC, Notch2-IC or Notch3-IC.

Expression of Deltex homologues in T cells.

To test whether Deltex family members are expressed during T cell development, we sorted thymocytes from C57BL/6 mice based on their expression of CD4 and CD8 into DN ($CD4^-/CD8^-$), DP ($CD4^+/CD8^+$) or SP (CD4 or CD8) subsets to generate cDNA. The expression of deltex family members was determined by TaqMan RT-PCR analysis and plotted as relative expression normalized to HPRT (Figure 25A). This analysis reveals that the mRNAs for all 3 Deltex homologues are highly expressed in thymocytes, although only Deltex1 exhibited the dynamic expression pattern that is characteristic of Notch responsive genes. To test more directly whether the Deltex mRNAs are induced by Notch signaling, we examined expression of Deltex family mRNAs in a thymoma cell line 1010 that had been transduced with retroviruses expressing empty vector, Notch1-IC or Notch3-IC (Figure 25B). While Hes1 and Deltex1 mRNAs were highly induced by Notch signals derived from either Notch1 or Notch3, expression of Deltex2 or Deltex4 was unchanged by Notch signaling.

Deltex homologues are found in different subcellular locations.

It is not clear how Deltex proteins regulate Notch signals. Some reports have proposed that Deltex can regulate the activity of Notch signals within the cytoplasm, potentially by acting on the full length Notch receptor upstream of Notch-IC[92]. Alternatively, it has been proposed that Detlex acts within the nucleus, altering Notch-mediated transcriptional activation by binding to both Notch-IC and the transcriptional coactivator p300[94]. To better understand how different Deltex homologues could participate in regulating Notch signals, we examined the subcellular localization of recombinant Deltex proteins in Cos-7 cells. Flag-tagged Deltex1, Deltex2 or Deltex4 were co-expressed with myc-tagged Notch-IC, and their subcellular localization was examined by immunofluorescence. The results of this analysis, shown in Figure 26, reveal that

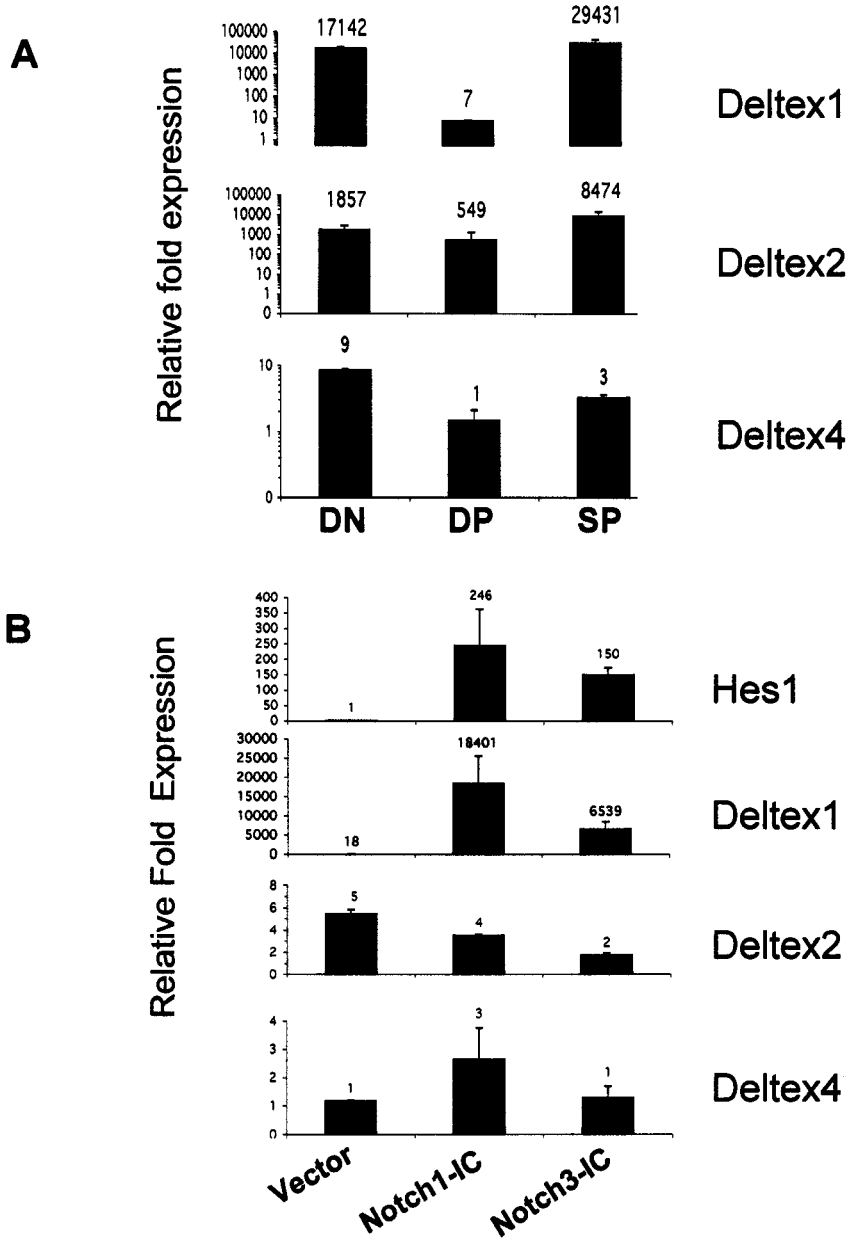


Figure 25: Deltex1, Deltex2 and Deltex4 are expressed in thymocytes, but only Deltex1 is induced by Notch signals.

(A) Expression of Deltex family members in sorted DN thymocytes by TaqMan RT-PCR. Relative fold expression is shown for cDNAs normalized to HPRT.
(B) Exression of Hes1, Deltex1, Deltex2 and Deltex4 in 1010 expressing Notch1-IC or Notch3-IC. Relative expression of each gene was normalized to HPRT.

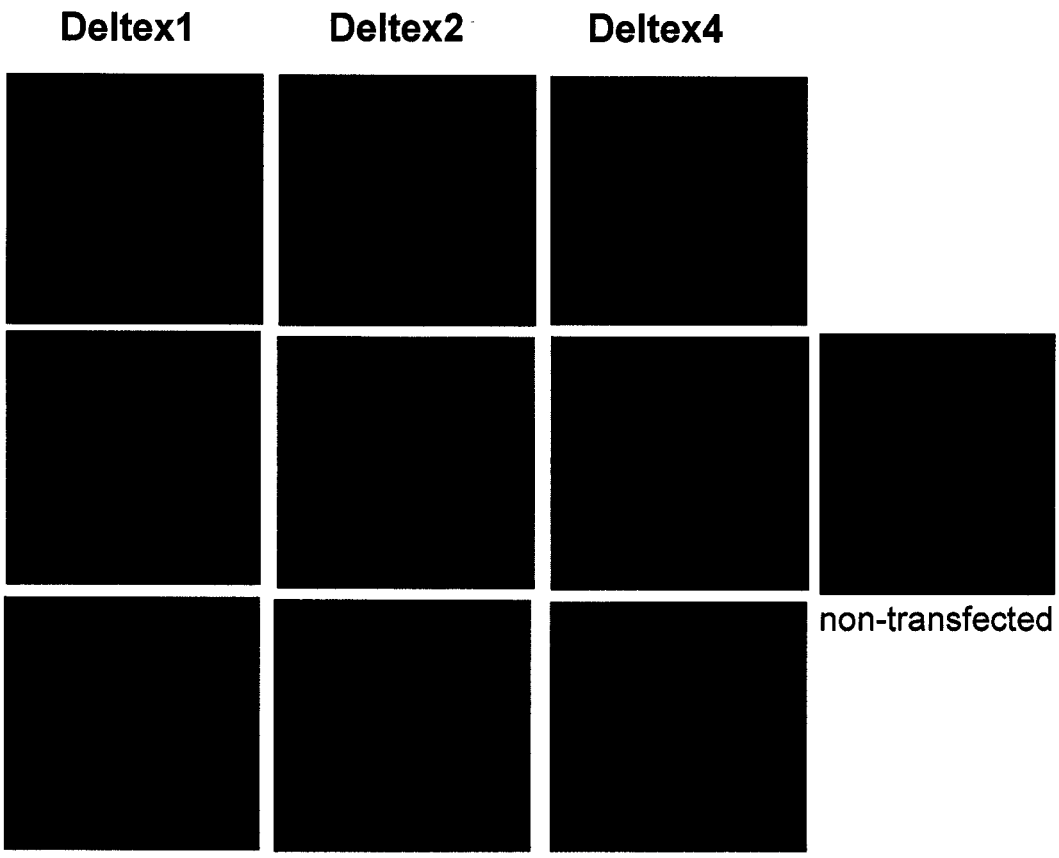


Figure 26: Deltex family members localize in different subcellular compartments.

Cos-7 cells were co-transfected with the indicated plasmids for Deltex family members and Notch1-IC. Immunofluorescent staining of myc-tagged Notch-IC (green) and flag-tagged Deltex family members (red) was performed 24 hours after transfection. Bottom panels show overlays of the two images above. Images shown at 20X magnification.

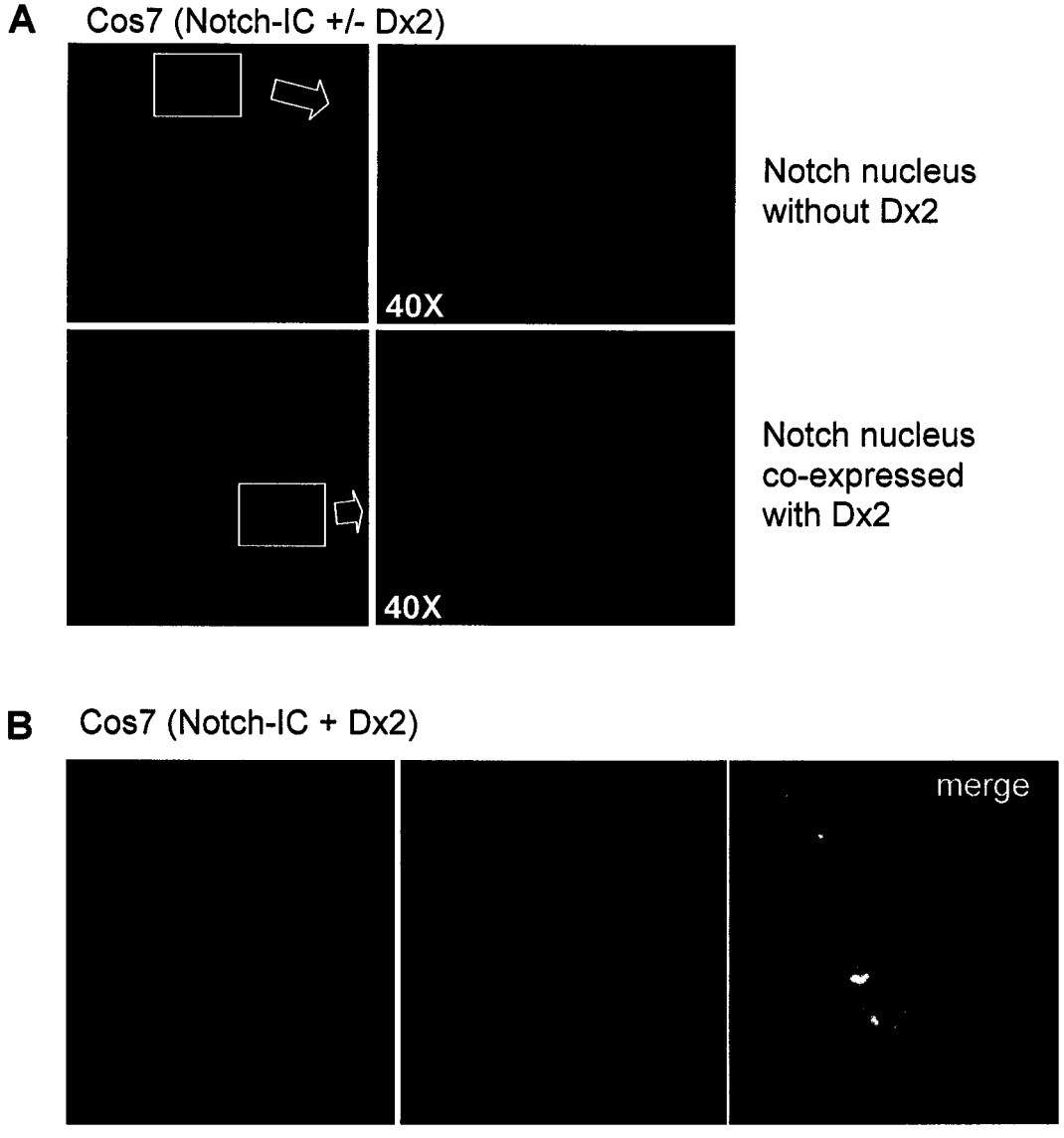


Figure 27: Deltex2 recruits Notch IC to PML bodies

(A) Localization of Notch-IC in Cos-7 expressing myc-Notch-IC alone, or co-expressing myc-Notch-IC and flag-Deltex2. (B) Cos-7 co-expressing myc-Notch-IC and flag-Deltex2 were stained with anti-flag (red) and rabbit anti-PML (green). Right panel shows overlay of red and green images. Images shown at 20X magnification unless indicated otherwise.

the three Deltex homologues are expressed in different regions within the cell. Deltex1 is expressed throughout the cytoplasm, whereas Deltex2 and Deltex4 are found mostly in the nucleus. We consistently observed that Deltex2 is localized within nuclear bodies that could also be detected by co-staining with antibodies to the promyelocytic leukemia antigen (PML)[113], and that Deltex2 appeared to recruit Notch-IC into nuclear bodies (Figure 27).

Deletion of Deltex1, Deltex2 and Deltex4 in T cells.

The above data demonstrating that Deltex1, Deltex2 and Deltex4 are expressed in thymocytes and that all three Deltex homologues are able to block Notch signals suggest that functional redundancy between the 3 Deltex homologues could mask any defects in Deltex1 deficient mice. To determine whether Deltex proteins play a role in regulating Notch signals during T cell development, we attempted to delete all 3 Deltex family members in T cell progenitors. First we generated Deltex1/Deltex2 double deficient mice by gene targeting, and then we attempted to inhibit expression of the Deltex4 mRNA in stem cells using RNA interference. We examined the ability of fetal liver stem cells that do not express Deltex1 or Deltex2 and have reduced levels of Deltex4 to differentiate into T cells using the OP9-Delta1 in vitro culture system described in Chapter 2 above.

First, we generated Deltex2 deficient mice by gene targeting. The first 2 coding exons of Deltex2 were replaced with the gene for neomycin flanked by recognition sequence for the FLP recombinase (Figure 28) and 96 ES cell clones were screened by Southern blot analysis. Two clones showing the targeted allele are shown in Figure 28B. Cells from clone #66 were injected into blastocysts, and the resulting chimeric mice were bred to mice that express the FLP recombinase to remove the neomycin gene[114]. Northern blot analysis of splenocytes from the resulting mice confirmed that the targeted Deltex2 allele produces a truncated mRNA. Although we were unable to detect the Deltex2 protein in normal splenocytes using a polyclonal antibody raised against the c-terminal region of Deltex2 (data not shown), the truncated Deltex2 mRNA

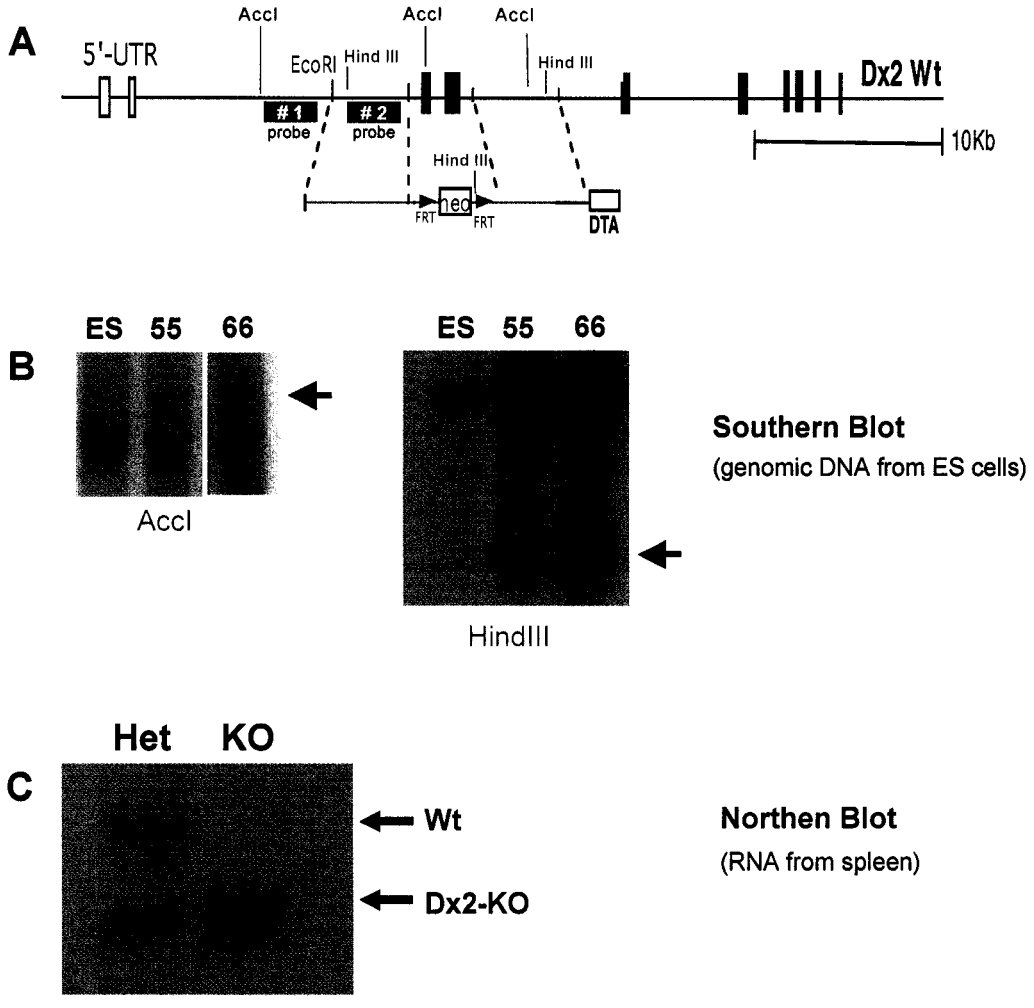


Figure 28: Targeted deletion of Deltex2.

(A) Targeting strategy for Deltex2-KO mice. The first 2 coding exons of Deltex2 were replaced with neomycin. (B) Southern blot on genomic DNA isolated from ES cells used to generate Deltex2-KO mice. Genomic DNA was digested with Acc1 (left, probe #1), or digested with HindIII (right, probe #2). (C) Deltex2 knockout mice produce a truncated mRNA. Northern blot on 10μg of total RNA isolated from spleen of a Deltex2 knockout (KO) or heterozygous littermate control (Het). Probe is localized within the 3' end of the Deltex2 coding region. Arrows indicate bands representative of the targeted allele.

produced in Deltex2-deficient mice is unlikely to code for a functional protein for 2 reasons. First, the initiating ATG is deleted. And second, even if a truncated protein is produced, it is unlikely to be functional because it lacks the first domain of Deltex2 which is essential for binding to Notch-IC (data not shown). Deltex2-KO mice were viable, and showed no overt developmental defects. We were also unable to detect any abnormalities in T or B cell development by FACS analysis for developmental markers (data not shown).

We bred the Deltex2-KO mice to our Deltex1-KO mice to generate double knockout mice (Dx1-KO/Dx2-KO). Since the genes for Deltex1 and Deltex2 are both found on chromosome 5 (Figure 29A), we bred homozygous Deltex1-KO mice with homozygous Deltex2-KO mice and bred the resulting double heterozygous progeny to C57BL/6 mice to identify those that had undergone homologous recombination such that both the Deltex1 and Deltex2 mutations were present on the same chromosome. This analysis revealed that homologous recombination within the 15 megabase region between the Deltex1 and Deltex2 genes occurred with a frequency of approximately 1 out of 15. Expression of the truncated Deltex1 and Deltex2 mRNAs in splenocytes from the Dx1-KO/Dx2-KO mice was confirmed by Northern blot analysis (Figure 29C). We were unable to detect any developmental abnormalities in the Dx1-KO/Dx2-KO mice, and preliminary analysis did not reveal any defects in T or B cell development by FACS analysis for developmental markers (data not shown). We were also unable to detect any differences in the level of Notch signaling, as detected by expression of the mRNAs for Hes1, Deltex1 or pre-Tα in thymocytes isolated from Dx1-KO/Dx2-KO mice (Figure 30).

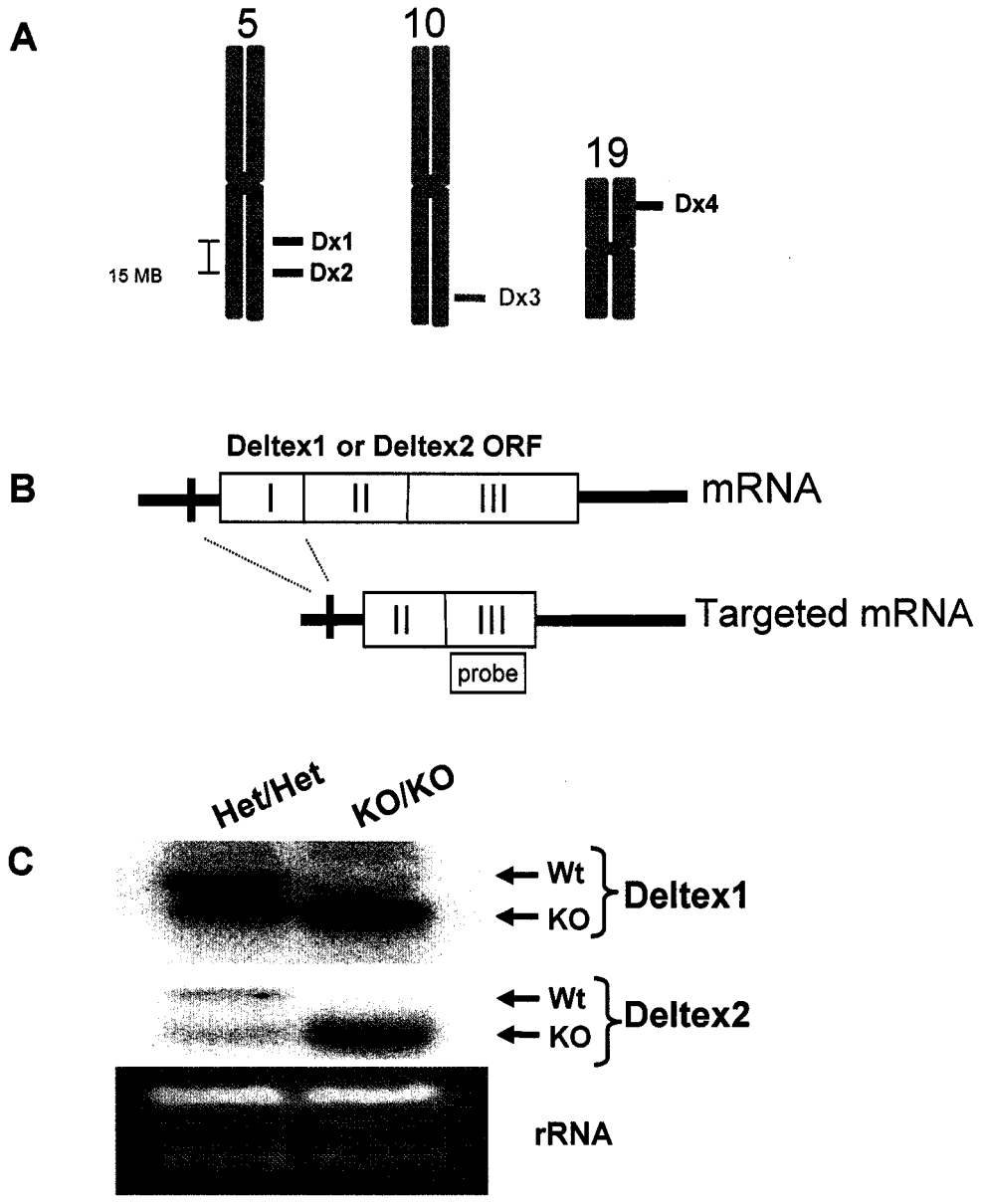
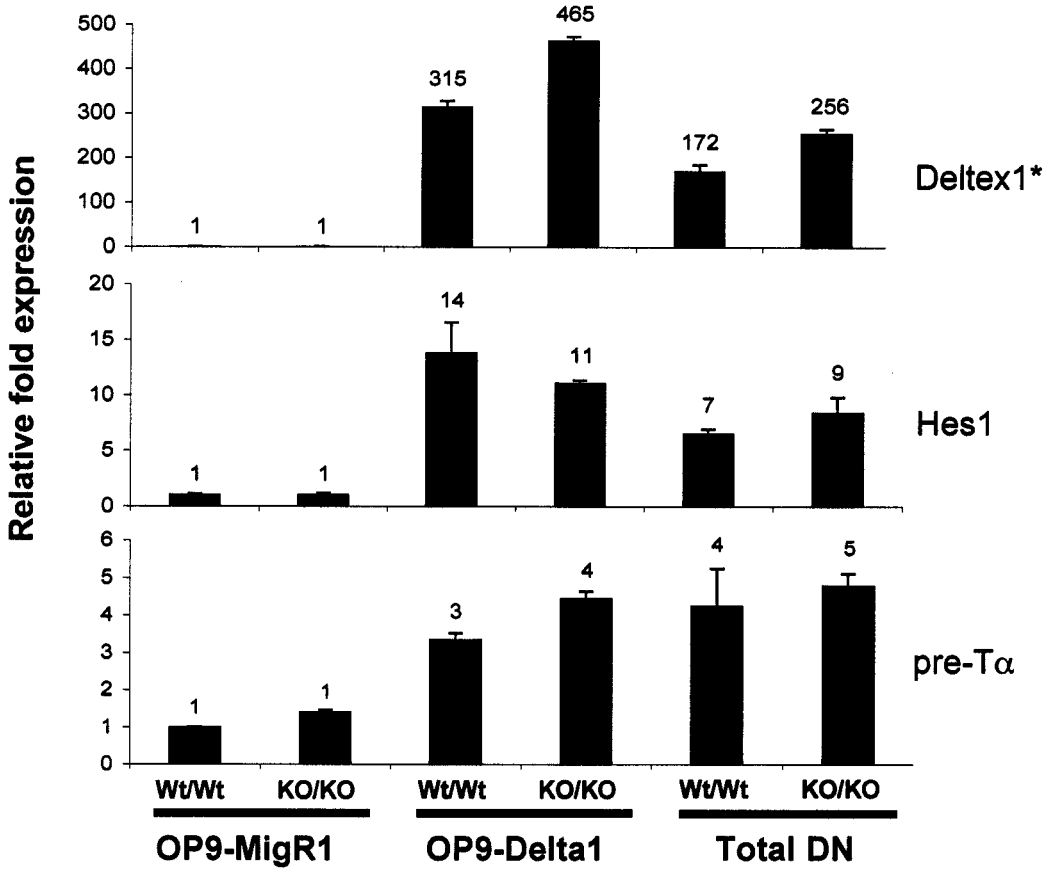


Figure 29: Generating Deltex1/Deltex2 double knockout mice

(A) Chromosomal location of murine Deltex homologues. (B) Predicted mRNAs for Deltex1 or Deltex2 in double knockout mice. Probes used for Northern blot analysis of Deltex1 or Deltex2 were designed to identify the 3' region of the open reading frame (ORF). (C) Northern blot on splenocytes from Deltex1/Deltex2 double heterozygous (Het/Het) or double knockout (KO/KO) mice.



*primers for Deltex1 were designed within the 3' region of the mRNA which is not deleted in Deltex1-KO mice.

Figure 30: Induction of Notch-responsive genes is normal in thymocytes from double knockout mice.

Expression of Notch responsive genes by TaqMan RT-PCR in DN4 thymocytes from Deltex1/Deltex2 double knockout (KO/KO) or wild type littermate control (Wt/Wt) mice. Sorted DN4 thymocytes were cultured for 24 hours on OP9-MigR1, OP9-Jag1 or OP9-Delta and stromal cells were removed by MACS selection for $CD45^+$ cells. Induction of Notch responsive genes is compared to the normal levels of Deltex1, Hes1 or pre-Tα found in the total $CD4^-CD8^-$ (Total DN) fraction of freshly isolated thymocytes taken from the same mice prior to cell sorting.

Finally, to eliminate expression of the final Deltex homologue, Deltex4, we generated two RNAi expression vectors[115] that each express a short hairpin RNA containing a 19 base pair sequence derived from the coding region of Deltex4 (Figure 31A). These vectors were used to generate retroviruses in the PhoenixE packaging cell line. Co-infection of stem cell populations isolated from fetal livers of C57BL/6 mice with retroviral supernatants containing the 2 Deltex4 RNAis resulted in a 50% reduction of the Deltex4 mRNA (Figure 31B). We then used the above retroviruses to express the Deltex4 RNAi in fetal liver stem cells isolated from Dx1-KO/Dx2-KO embryos. Mice that were heterozygous for both Deltex1 and Deltex2 were bred together, and fetal liver was isolated from the resulting embryos and typed for Deltex1 and Deltex2 by PCR analysis on genomic DNA. Cells from wild type (Wt/Wt) or Dx1-KO/Dx2-KO (KO/KO) embryos were pooled and infected with either the empty RNAi vector (LTRH) or a pool of the 2 Deltex4 RNAi vectors (RNAi). After 48 hours the resulting stem cells were sorted for expression of cKit and the human-CD4 marker protein which identifies cells that had been effectively transduced with the retrovirus (Figure 32).

Notch signals are slightly more potent in Deltex-deficient T cell progenitors.

Since our above analysis (Figure 17 and 24) suggested that Deltex proteins can inhibit Notch signals, we hypothesized that Notch signals delivered through the natural ligands Delta1 or Jagged1 should be more potent within mutant T cell progenitors that express reduced levels of Deltex1, Deltex2 and Deltex4. Our previous analysis, presented in Chapter 2 also suggested that Notch signals delivered through OP9-Jagged1 are less potent than signals delivered through OP9-Delta1, and that modulation of the dose of Notch signals delivered to T cell progenitors can affect their ability to differentiate into the T cell, B cell or NK cell lineages. To test the hypothesis that Notch signals are more potent in the absence of Deltex, we examined the differentiation of the fetal liver stem cells shown in Figure 32 in the presence of different doses of Notch signals

and examined 2 Notch functions: inhibition of B cell development and the promotion of T cell development.

First we examined whether Notch signals could inhibit B lineage commitment equivalently in the above stem cells that do or do not express Deltex proteins. Fetal liver stem cells differentiated efficiently into $CD19^+$ B cells on OP9-MigR1, whereas B cell development was completely inhibited when these cells were cultured on OP9-Delta1 (Figure 33). When we added increasing doses of the presenilin inhibitor X to OP9-Delta1 cultures, Notch signals were partially blocked and fetal liver stem cells from wild type mice expressing the empty vector (Wt/Wt-LTRH) or expressing the Deltex4 RNAi (Wt/Wt-RNAi) were able to differentiate into either B cells or NK cells on OP9-Delta1. In contrast, we were not able to completely inhibit Notch signals in the double knockout (KO/KO-LTRH) or triple deficient (KO/KO-RNAi) stem cells using the presenilin inhibitor. These data suggest that Notch signals are slightly more potent in the absence of Deltex. However, the increased Notch signaling observed in our Deltex-deficient T cell progenitors was not sufficient to permit the differentiation of these cells into the T cell lineage in response to the weaker Notch signals provided by OP9-Jagged1 (Figure 34).

Finally, we tested whether Notch signals could promote T cell development equivalently in the above stem cells. Notch signals promote the maturation of T cell progenitors through the DN to DP stages of thymocyte development and are essential for promoting the proliferative expansion of thymocytes throughout these stages. T cell maturation was largely unchanged in Dx1-KO/Dx2-KO progenitors expressing the RNAi for Deltex4. Although, in 2 separate experiments we observed a slight increase in their maturation toward the DP stage after 14 days culture on OP9-Delta1 (Figure 35). Since Notch signals are essential for promoting the proliferative expansion of DN3 thymocytes that have undergone β-selection, we predicted that if Notch signaling is more potent in the absence of Deltex molecules then the proliferation of T cell progenitors should be enhanced in the Dx1-KO/Dx2-KO progenitors expressing the Deltex4 RNAi. In contrast we found that in cultures of Dx1-KO/Dx2-KO stem

cells expressing the Deltex4-RNAi the total number of T cells recovered after 6, 14 or 20 days was similar or reduced compared to wild type controls. We calculated that the reduced cell recoveries seen in Figure 36 could be accounted for if the overall recovery of Dx1-KO/Dx2-KO T cells expressing the Deltex4 RNAi (KO/KO-RNAi) was approximately 96% of the wild type (Wt/Wt-LTRH) controls at every cell division.

Together, the above data examining the effect of Deltex deficiency on Notch signaling during T cell development reveal that although over-expression of Deltex in T cell progenitors can inhibit Notch signals the normal levels of Deltex1, Deltex2 and Deltex4 found in thymocytes do not appear to have an important role in regulating Notch signals during T cell development.

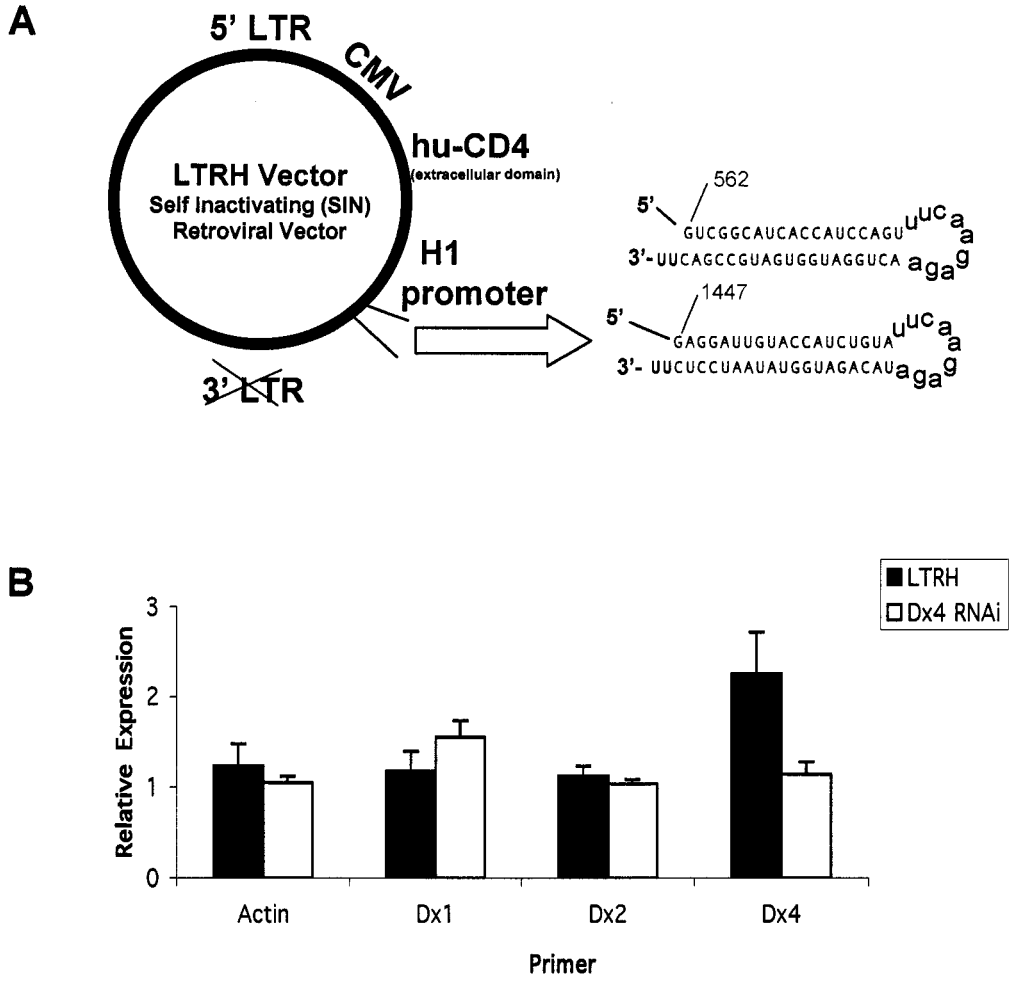


Figure 31: Inhibition of Deltex4 mRNA by RNA interference.

(A) LTRH vector used for retroviral expression of short interfering RNAs for Deltex4 (Dx4 RNAi). The extracellular domain of human CD4 is expressed under the control of the CMV promoter so that cells expressing the RNAi can be identified by staining for human CD4. Hairpins generated for the 2 Deltex4-RNAi's used (beginning at position 562 and 1447 within the coding region of Deltex4) are shown. (B) Inhibition of Deltex4 mRNA in stem cells from fetal liver. Fetal liver cells from day 14 embryos were transduced with empty vector (LTRH) or co-infected with both Deltex4 RNAi's shown in (A), and RNA was isolated after 4 days culture. Expression of Deltex1, Deltex2, Deltex4 and β-actin was determined by TaqMan RT-PCR analysis. Relative cDNA expression was determined for cDNAs normalized to HPRT.

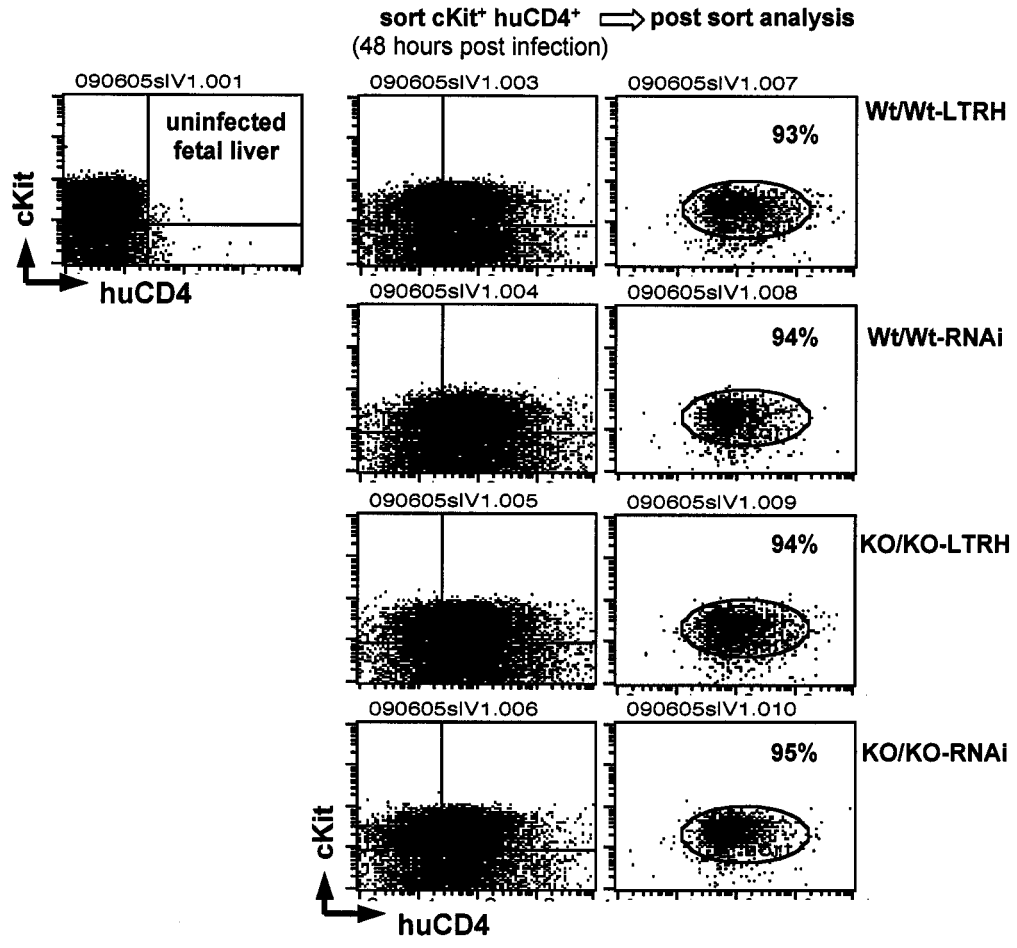


Figure 32: Post sort analysis on fetal liver stem cells from Deltex1/Deltex2 double knockout embryos expressing the Deltex4 RNAi.

Fetal liver from day 14 embryos was genotyped by PCR, and cells from 3 Wt and 3 Dx1/Dx2 double KO livers was pooled. Lineage positive cells were depleted by negative selection on MACS beads using biotinylated antibodies for Ter119, Gr-1, Mac-1 and B220. The resulting cells were infected with retroviruses expressing the RNAi vector alone (LTRH) or a pool of 2 Deltex4 RNAi's (RNAi) in the presence of IL-7 and SCF. After 48 hours cKit+/human-CD4+ stem cells were sorted and plated on OP9 stromal cells with Flt3L and IL-7. Cells were counted 3 times to assure equal numbers of stem cells were plated on Day 0. Cells were plated at 10,000 cells per well unless stated otherwise.

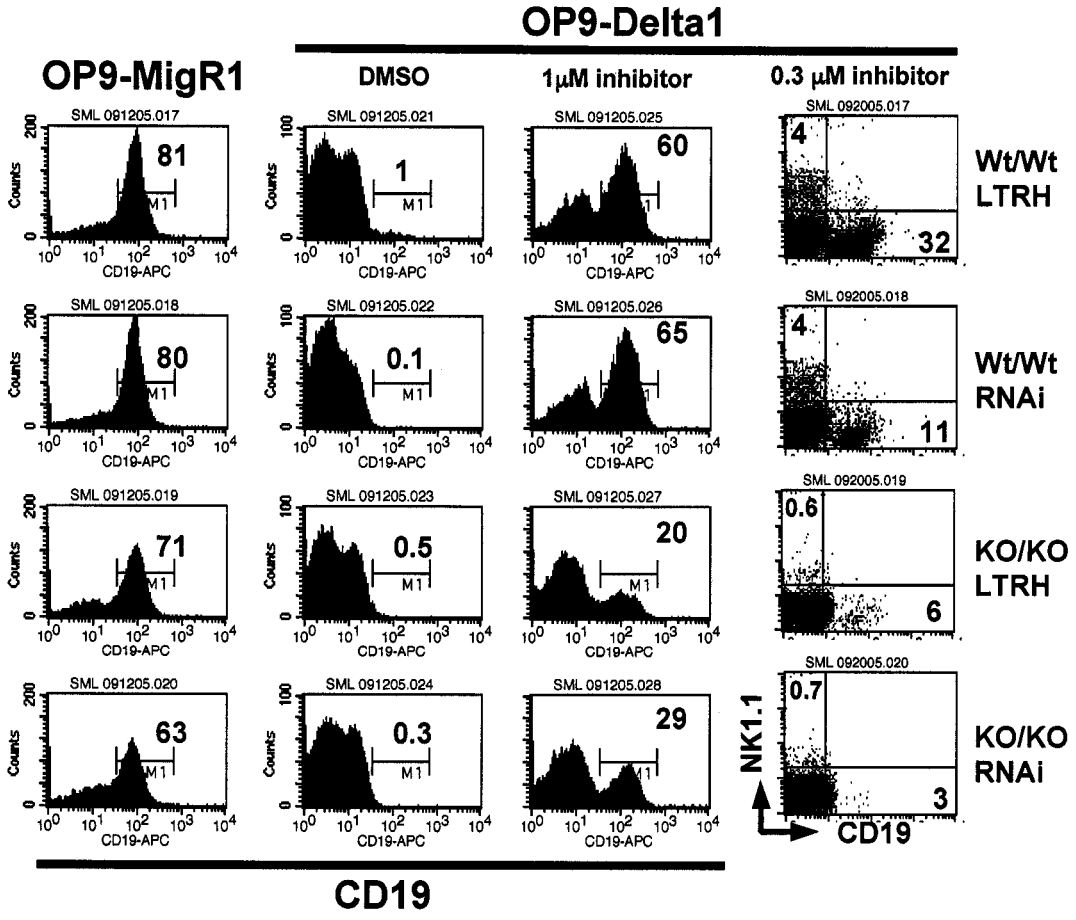


Figure 33: Notch signals are more potent in Dx1/Dx2 double KO precursors.

Expression of CD19 on fetal liver stem cells derived from wild type (Wt/Wt) or Deltex1/Deltex2 double knockout (KO/KO) embryos infected with empty vector (LTRH) or Deltex4 RNAi (RNAi). Day 14 embryos were typed for Deltex1 and Deltex2 by PCR on genomic DNA, infected with the indicated retroviruses and cultured in the presence of SCF and IL-7 for 2 days. Lineage negative, human $CD4^+$ stem cells were sorted and plated at 2,000 cells per well on OP9-MigR1 or OP9-Delta1 in the presence of carrier (DMSO) or increasing doses of presenilin inhibitor. Expression of cell surface markers was assessed by FACS analysis on day 6 (histograms) or day 14 (dot plots).

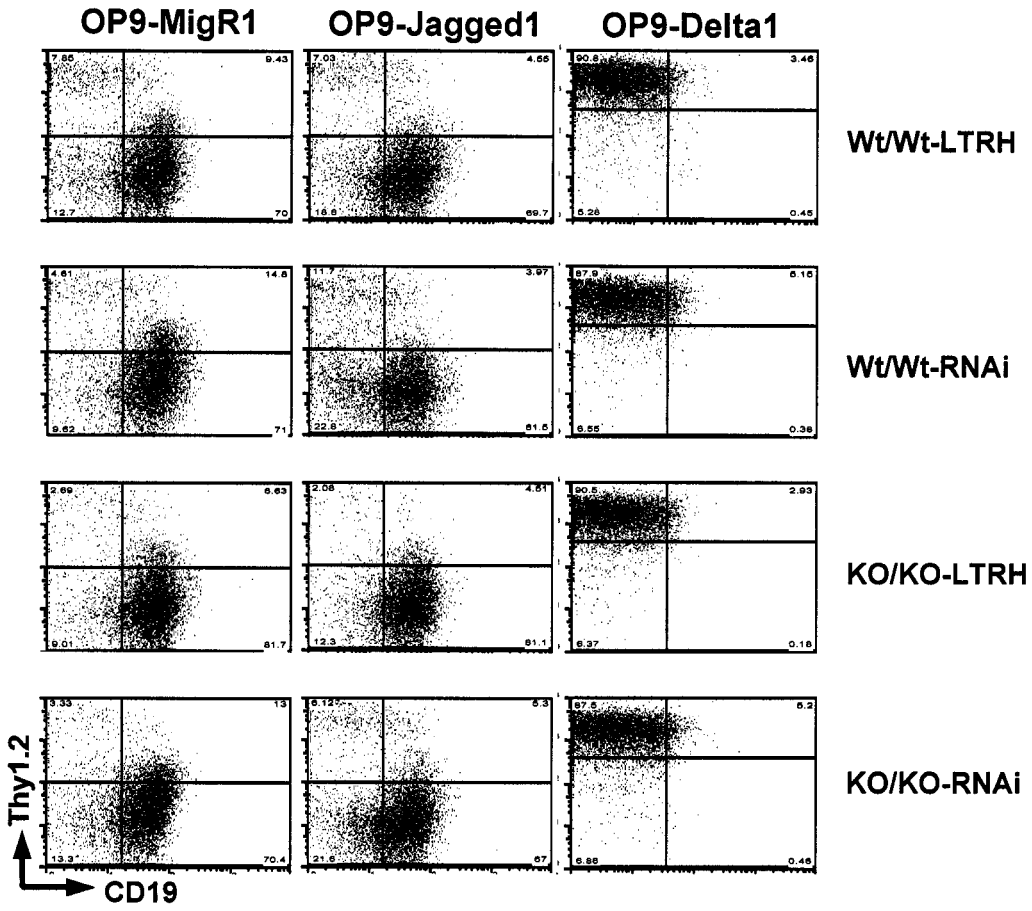


Figure 34: Inhibition of all three Deltex family members in Deltex1/Deltex2 double KO stem cells expressing Deltex4 RNAi does not permit T cell development on OP9-Jagged.

Expression of T lineage (Thy1.2) or B lineage (CD19) markers on fetal liver stem cells derived from wild type or Deltex1/Deltex2 double knockout mice infected with empty vector (LTRH) or Deltex4 RNAi (RNAi). Fetal liver from Day 14 embryos was typed for Deltex1 and Deltex2 by PCR on genomic DNA, infected with the indicated retroviruses and cultured in the presence of SCF and IL-7 for 2 days. Lineage negative, human $CD4^+$ stem cells were sorted and plated at 2000 cells per well on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. Expression of cell surface markers was assessed by FACS analysis on day 14.

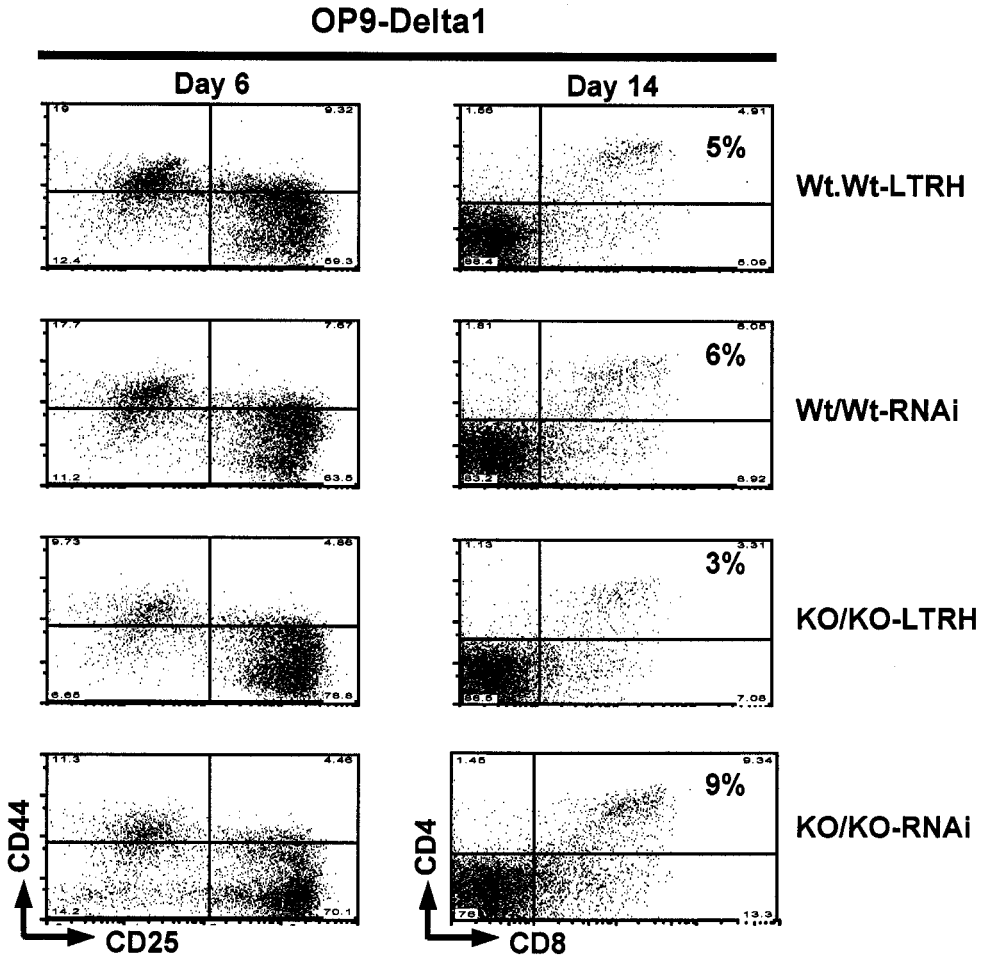


Figure 35: T cell development is normal in Deltex1/Deltex2 double KO stem cells expressing Deltex4 RNAi.

Expression of T lineage markers on fetal liver stem cells derived from wild type or Deltex1/Deltex2 double knockout mice infected with empty vector (LTRH) or Deltex4 RNAi (RNAi). Fetal liver from Day 14 embryos was typed for Deltex1 and Deltex2 by PCR on genomic DNA, infected with the indicated retroviruses and cultured in the presence of SCF and IL-7 for 2 days,. Lineage negative, human $CD4^+$ stem cells were sorted and plated at 2000 cells per well on OP9-MigR1, OP9-Jagged1 or OP9-Delta1. Expression of cell surface markers was assessed by FACS analysis on day 6 and day 14.

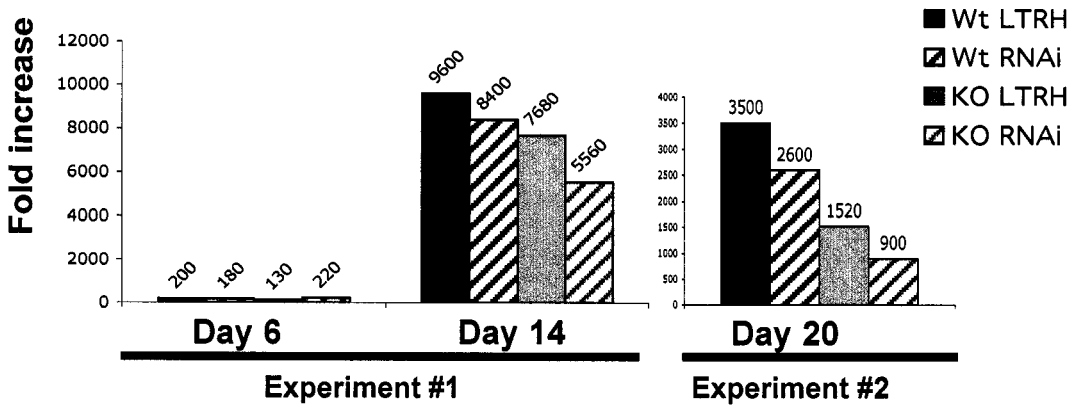


Figure 36: Notch-dependent expansion of fetal liver stem cells is reduced in the absence of Deltex.

Experiment #1: Total cells recovered per well from the experiment shown in Figure 33. Fetal liver stem cells were infected with empty vector (LTRH) or Deltex4 RNAi (RNAi). cKit$^+$/huCD4$^+$ cells were sorted and counted to assure equal numbers of cells were seeded on OP9-Delta on day 0. Data is shown as fold increase on day 6 from wells seeded at 10,000 cells/well, or on day 14 from wells seeded at 500 cells/well. Limiting dilution analysis showed similar numbers of cells were plated for the 4 samples.
Experiment #2: Total cells recovered on day 20 from the experiment shown in Figure 35. Stem cells were plated at 2000 cells per well on day 0, and the total DN fraction was purified on day 14 by depleting CD4 and CD8 cells using DYNAL beads. DN cells were labeled with CFSE and re-plated on fresh OP9 stromal cells . The total number of cells recovered per well after 6 additional days in culture is shown as fold increase from day 0. CFSE dilution was equivalent in the four samples (not shown). Numbers above bars indicate fold increase from Day 0.

Chapter V: Conclusions

Notch signals are essential for promoting T cell development. In this study we examined how regulation of Notch signals through 3 kinds of molecules: Jagged, Delta and Deltex influences T cell development. We showed evidence that 2 classes of Notch ligands, Jagged and Delta, transmit distinct signals to T cell precursors. This analysis revealed that Notch signals promote T cell development in 2 separable stages that can be distinguished by their ability to respond to the 2 classes of Notch ligands. In the first stage, Notch signals act on lymphoid precursors to inhibit B cell development. This function appears to require weaker Notch signals that can be transmitted by either Jagged or Delta. In the second stage, Notch signals act on T cell precursors to promote the expansion of immature T cells that have received a signal through the pre-T cell receptor, and this function requires potent Notch signals that can only be transmitted by Delta. Our data also reveal that differential signals emanating form the Jagged or Delta class of Notch ligands can influence the differentiation of immature thymocytes into either the NK or $\gamma\delta$ *vs.* $\alpha\beta$ T cell lineages. These data support a body of existing evidence that the dose of Notch signals delivered to thymocytes is important.

We also examined the effect of another Notch regulator Deltex on T cell development by generating Deltex knockout mice. There is significant evidence that the expression of Deltex1 is highly regulated during the early stages of T cell maturation and that over-expression of Deltex in T cell progenitors can inhibit Notch signals. Therefore, we hypothesized that Deltex would have an important role in regulating Notch signals during T cell development. However, we were unable to detect any defects in thymocyte development in our Deltex deficient mice.

Nevertheless, analysis of Notch signaling in T cell progenitors isolated form our Deltex knockout mice did provide some clues regarding how Notch signals are regulated through Deltex. When we examined the intensity of Notch signals within T cell progenitors that lacked Deltex1 and Deltex2 and expressed

reduced levels of Deltex4 using a sensitive in vitro assay we could detect that Notch signaling is slightly elevated in the absence of Deltex. These data suggest that our original hypothesis, that Deltex acts as a negative regulator of Notch signals in T cells was somewhat correct. However, our results also suggest that regulation of Notch signals through Deltex is complex and involves a process that is not a simple matter of tuning the overall dose of Notch signals. Indeed, Deltex appeared to have opposing effects when we examined the two different types of Notch functions that regulate T lineage commitment *vs.* the proliferation of immature thymocytes.

The observation that Deltex can have opposing effects on Notch signals in different settings was also evident in another experimental system that examines Notch induced T cell leukemia. When we examined the severity of Notch induced leukemia in Deltex1 knockout mice (Figure 23), the disease appeared more severe in Deltex knockout mice when Notch-IC was expressed in bone marrow stem cells, but less severe when Notch-IC was expressed using transgenic Lck-Notch-IC mice in which expression of Notch-IC is activated during the DN2/DN3 stage. These results suggest that in the Deltex knockout cells Notch signals are more potent when Notch-IC is expressed in bone marrow, but less potent when Notch-IC is expressed in thymocytes. These data show a similar contradiction to that shown in Figure 33 and 36. Notch signals appear more potent in Deltex knockout cells when we examine B lineage commitment, but less potent when we examine proliferation. It is difficult to interpret how the above data obtained in artificially manipulated systems will translate to the role of Deltex in vivo. Nevertheless, it is interesting to note that Deltex has been proposed to behave as a negative or positive[90,116] regulator of Notch signaling in different settings. Therefore, we look forward to future studies examining the role of Deltex in regulating Notch signals during different developmental stages in other systems that we were not able to address here.

Methods

Immunohistochemistry

Thymic tissue was frozen in OCT compound (Sakura Finetek, Torrance, CA), cut into 6-8 micron sections, and collected on Frost Plus microscope slides (VWR Scientific, West Chester, PA). After air drying for at least two hours, the tissue was immersed for 20 minutes in cold acetone (-20°C), rinsed in phosphate buffered saline (PBS), and then processed for immunofluorescence microscopy. Sections were stained with a cocktail of primary antibodies diluted in neat culture supernatant from either the ER-TR5[71] (a generous gift of W. Van Ewijk) or CDR-1[72] (American Type Culture Collection (ATCC, Rockville, MD) hybridomas. Expression of Notch ligands was detected using the following polyclonal anti-sera from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Jagged1 (sc-6011), Delta1 (sc8155) and Jagged2 (sc8158). To determine specificity, antibodies were pre-incubated with their respective immunizing peptides. Anti-sera directed against Delta1 and Jagged2 revealed staining on cortical epithelial cells (data not shown), but the signal was not inhibited by pre-incubation with the immunizing peptides. Flurochrome-conjugated anti-Ig secondary antibodies chicken anti-goat IgG-Alexa 488 and rabbit anti-rat IgG-Alexa 546 conjugates were purchased form Molecular Probes (Eugene, OR). Secondary antibodies were diluted in PBS containing 10 mg/ml BSA (Sigma Chemical Co., St. Louis, MO) and 10% normal mouse serum.

Generation of OP9 stromal cells expressing Jagged1

The full length cDNA for murine Jagged1 (accession BC058675) was obtained from ATCC (Image clone 6834418), cloned into the EcoRI site of MigR1 (a kind gift from Dr. W. Pear, University of Pennsylvania, Philadelphia, PA), and the sequence of the coding region of Jagged1 was confirmed by dideoxy sequencing. The resulting expression vector encodes a bi-cistronic transcript for Jagged1 and green fluorescent protein (GFP) separated by an internal ribosomal

entry sequence (IRES). Retroviral supernatants were generated in the Phoenix ecotropic packaging cell line as described previously[49], and used to generate OP9 stromal cells expressing empty vector (MigR1) or Jagged1. GFP^+ cells were purified by cell sorting, and expression of Notch ligands in the parental, or OP9-Jagged1 stromal cells was confirmed by RT-PCR. RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturers instructions, and cDNA was generated using M-MuLV reverse transcriptase (Fermentas, Hanover, MD). cDNAs were normalized by TaqMan PCR (PE Applied Biosystems, Foster City, CA) for HPRT, as described previously[61]. PCR was carried out on normalized cDNAs using Taq polymerase (Perkin Elmer) and the following primers:

Jagged1:

(5'-TGGTAGACAGAGAGGAGAAGG and 5'-TCAATTTCCCAGCCAACC),

Jagged2:

(5'-TGGAAACAGTTGTTATGGGTG and 5'-GGTGAACTTGTGTGAGATGAACT),

Delta1:

(5'-ACCTCGGGATGACGCCTTTG and 5'-AGACCACCACAGCAGCACAG),

Delta4:

5'-GCACCAACTCCTTCGTCGTC and 5'-TCACAAAACAGACCTCCCCA) and

G3PDH: (5'-TGAAGGTCGGTGTGAACGGATTTGGC and

5'-CATGTAGGCCATGAGGTCCACCAC).

The identity of PCR products was confirmed by Southern Blot hybridization, and relative expression level was estimated by comparing the level of input cDNA (5-fold serial dilutions) resulting in equivalent hybridization signals. Expression of Jagged1 protein was detected by flow cytometric analysis (CellQuest Pro software, BD Biosciences, San Diego, CA) using a goat polyclonal antibody directed against the extracellular domain of rat Jagged1 (R&D Systems, Minneapolis, MN), or by immunoprecipitation using the above antibody, followed by Western Blot using a goat polyclonal antibody specific for the intracellular domain (sc6011) of rat Jagged-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

C2C12 differentiation cultures

C2C12 myoblasts (ATCC, Rockville, MD) were maintained in DMEM containing 10% fetal calf serum (Gibco/Invitrogen, Carlsbad, CA), and 5% cosmic calf serum (Hyclone, Logan, UT). C2C12 cells were plated in 24 well culture dishes at 5 X 10^3 cells/well on monolayers of OP9 stromal cells that had been plated at 2.5 X 10^4 cells/well on the previous day. C2C12 differentiation was induced by replacing the culture medium with differentiation media, DMEM 10% horse serum (Gibco). The γ-secretase inhibitor X (Calbiochem, San Diego, CA) or 0.1% DMSO carrier was added to selected cultures, and the growth medium was replaced every 2-4 days. To monitor differentiation, RNA was isolated from a single well to generate cDNA and cDNAs were normalized by TaqMan for HPRT. The degree of C2C12 differentiation was monitored by RT-PCR for MLC2 as described previously[73].

Isolation of stem cells

Stem cells were isolated from 4-12 week old C57BL/6 mice (Taconic, Germantown, NY). For BM-HSCs, bone marrow was pre-depleted with biotinylated antibodies for B220 (RA3-6B2), Mac1 (M1/70) and Gr-1 (8C5), using streptavidin-conjugated magnetic beads (Dynal Biotech, Brown Deer, WI), according to the manufacturer's instructions. Cell suspensions were incubated with Fc-block (24G2) prior to staining for cell surface markers. Cells were stained for surface expression of lineage (lin) markers using PE-conjugated antibodies to the following antigens Thy1.2 (53-2.1), B220 (RA3-6B2), Mac1 (M1/70), Gr1 (8C5), CD11c (HL3), Ly-76 (Ter-119), NK1.1 (PK136), TCRβ(H57-597) and TCRδ(GL3), and lineage positive cells gated out. HSCs were identified as lin^- $cKit^{hi}$ (2B8) $Sca1^{hi}$ (E13-161.7) For thymus DN subsets, thymocytes were pre-depleted with biotinylated antibodies for CD4 (RM4-5) and CD8 (53.67), and lineage marker positive cells were gated out as described for BM-HSC, except Ter119, Gr1 and Thy1.2 antibodies were omitted and T cells were excluded with CD4 and CD8. DN1, DN3 and DN4 thymocytes were identified by expression of

CD44 (IM7) and CD25 (7D4). All stem cells were sorted using a FACS Vantage Cell sorter (BD Biosciences, San Diego, CA), and antibodies were obtained from Pharmingen (BD Biosciences, San Diego, CA).

Cell culture for In vitro T cell development

Parental OP9 stromal cells, and cells expressing Delta-1 were a kind gift from J. C. Zuniga-Pflucker, University of Toronto, Ontario Canada). In vitro T cell development cultures were carried out as described previously[61,68]. OP9 monolayers were prepared one day in advance by plating stromal cells at 2.5 x 10^4 cells/well in 24 well culture dishes, and stem cell populations were plated at 1000 and 10,000 cells per well onto OP9 monolayes in RPMI media (Gibco) containing 10% FCS, supplemented with L-glutamine, β-mercaptoethanol, penicillin/streptomycin and gentamycin. Growth media was supplemented with 5 ng/ml recombinant IL-7 and Flt3L (PeproTech, Rocky Hill, NJ). The γ-secretase inhibitor X (Calbiochem, San Diego, CA) or 0.1% DMSO carrier was added to selected wells on day 0 and replaced every 3-4 days.

Flow cytometric analysis for cell differentiation markers.

Expression of cell differentiation markers was analyzed by 4 color flow cytometry using a FACSCalibur (BD Pharmingen). Cells were incubated with Fc-block (24G2), and stained using the following antibodies B220-CyC (RA3-6B2), CD19-bio (ID3), CD44-FITC (IM7) CD25-PE (7D4), CD4-CyC (RM4-5), CD8-APC (53.67), NK1.1-PE (PK136), CD11c-FITC (HL3), TCRβ-FITC (H57-597), TCRδ-PE (GL3).

Primers and probes for TaqMan analysis

Hes1:

For: 5'-TACCCCAGCCAGTGTCAACA

Rev: 5'-TTCTTGCCCTTCGCCTCTT

Probe: 5'-TGAGCACAGAAAGTCATCAAAGCCTATCATGG

pre-Tα:

For: 5'- CTGCTTCTGGGCGTCAGGT

Rev: 5'- TGCCTTCCATCTACCAGCA

Probe: 5'- CCTTTCCGTCTCTGGCTCCACCCA

p21/Waf1:

For: 5'-GGGTGGGCCCGGAA

Rev: 5'-TGATAGAAATCTGTCAGGCTGGTC

Probe: 5'-ATCTCAGGGCCGAAAACGGAGGC

Deltex1:

For: 5'-TGAGGATGTGGTTCGGAGGT

Rev: 5'-CCCTCATAGCCAGATGCTGTG

Probe: 5'-CGCCTGATGAGGACTGTACCATTTGCAT

Deltex2:

For: 5'-CCCCTTACATCATCGACCTCC

Rev: 5'-GCGCACAGACCTCATGGTG

Probe: 5'-CAGCTGGACTCAGTTTCGCCAGAACACT

Deltex4:

For: 5'- GGGATTCTATAGTAAAGGCATGGC

Rev: 5'- TCTATGTCCATTAGGGTCCAAGTTT

Probe: 5'- TCTCACCTTTGCCAGCCCATCTCGTAA

Notch reporter assay

The pGL2–8xCBF1 plasmid (a gift from Dr. S. D. Hayward, Johns Hopkins University, Baltimore, MD) was modified such that luciferase was replaced with GFP and introduced into Cos-7 cells. Plasmids containing the intracellular domain of Notch1, Notch2 and Notch3 fused to the myc tag, or the entire coding region of Deltex1, Deltex2 or Deltex4 fused the the flag tag were introduced by transient transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. GFP expression was monitored by FACS analysis after 24 hours. The remainder of cells used for FACS analysis were lysed in RIPA buffer and analyzed by Western Blot using antibodies to Flag (M2: Statagene) or Myc tag (9B11: Cell Signaling). The expression vectors were

constructed using the pCMV-Tag vectors (Stratagene) by fusing the appropriate coding regions as follows: Notch1: (Accession# NM008714 from 5329-8082), Notch2: (Accession# D32210 from 5262-7579), Notch3: (NM 008716 from 5064-7944) were fused in frame into CMV-Tag3. The entire coding regions of Deltex1: (NM_008052), Deltex2: (NM_023742) and Deltex4: (NM_172442) were fused into CMV Tag2. The epitope tag was cloned at the N-terminus in the above vectors.

Targeted Deletion of Deltex1 and Deltex2

The genomic DNA for Deltex1 (NCBI GeneID: 14357) and Deltex2 (NCBI GeneID: 74198) was obtained by screening a BAC library (Research Genetics RPCI-22 from the 129 mouse strain) using cDNA probes encompassing the first (Deltex1) or the first 2 coding exons (Deltex2). Genomic DNA was subcloned into pBluescript (Stratagene) and the Neo cassette flanked with FRT recombinase recognition sequences was subcloned from PGK-neo FRT2-Lox2 DTA, a kind gift from Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, Washington). Targeting vectors were introduced into TC1 ES cells (derived from 129 mice), and neomycin resistant clones were screened by Southern blot. For Deltex1-KO mice the targeted region, including the neomycin cassette were deleted by transient transfection of a plasmid encoding Cre recombinase (PGK-Cre). Knockout mice were generated from ES cells using established methods[98]. For Deltex2-KO mice the neo cassette was deleted by breeding mice to Flipper mice[114]. Deltex1/Deltex2 double knockout mice were generated by breeding the two strains together.

Typing Deltex1 and Deltex2 knockout mice

PCR analysis for typing genomic DNA.

Flipper PCR: 3 primer PCR detects Wt (500 bp) or FLP (250 bp).

R1295: 5'-GCGAAGAGTTTGTCCTCAACC)

R523: 5'-GGAGCGGGAGAAATGGATATG

R26F2: 5'-AAAGTCGCTCTGAGTTGTTAT

Deltex1 PCR: 3 primer PCR detects Wt (280 bp), 2Lox (314 bp) or KO (494 bp).

F6: 5'-TGACAGCCTGGGGTATGATGC

20R: 5'-CGGCCTGAGTGGTGGTAGATC

B3: 5'-GGTGGACGGGGAAGACTTTCTG

Deltex2 PCR: 3 primer PCR detects Wt (631 bp) or KO (300 bp).

F1: 5'-GATAGGGATCAAGAGTTGATC

R1: 5'-GGTCTTACCAGTGTTCTGGCG

R2: 5'-CTCCACCTGGCTTTGCATGAG

Southern blot on genomic DNA

Deltex1: 10μg of genomic DNA from ES cells was digested with KpnI and the Southern blot was probed with a genomic probe encompassing the region indicated in Figure 18. Further analysis using restriction sites and probes outside of the targeted region confirmed that the endogenous gene is altered in the Dx1-KO ES cells (not shown). Deltex2: Southern blots were preformed as described above using 10μg of genomic DNA from ES cells digested with AccI and probed with probe #1, or digested with HindIII and probed with probe #2 as shown in Figure 28.

Mixed bone marrow chimeras

Bone marrow from 8wk old donor mice, 2 Deltex1 knockout (Dx1-KO) or 2 homozygous wild type littermates (Wt) (both Ly5.2), was pooled and mixed at a 1:1 ratio with wild type competitor marrow from Pep3^b/Boy (Ly5.1) mice (Jackson Labs, Bar Harbor, ME). Bone marrow cells were depleted of mature T cells by complement lysis using antibodies to CD4, CD8 and Thy1.2. The donors shown in Figure 19 were back crossed 2 times to C57BL/6. The marrow was transferred into lethally irradiated recipients as follows: Recipients B6.SJL-*Ptprc* Pep3^b/Boy (Ly5.1/5.2: Jackson Labs, Bar Harbor, ME) were irradiated 1000 rads, and placed on antibiotic water one day prior to and for 3 weeks following bone marrow transfer. After 3 months the hematopoietic cells derived from competitor (Ly5.1) or Deltex1-KO littermates (Ly5.2) were detected using the following antibodies: CD45.1 (clone A20), or CD45.2 (clone 104), (BD Pharmingen).

Proliferation of mature T cells was measured by dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE: Molecular Probes Inc. Eugene, OR). For CFSE labeling, cells were washed in PBS containing 0.1% BSA and incubated at 37° C for 7 minutes in PBS containing 10μM CFSE. The reaction was stopped by adding cold media containing 1% BSA. The labeled cells were washed 3 times in complete media and stimulated with plate bound anti-CD3ε at 1μg/ml or 0.2μg/ml. Proliferation was measured as CFSE dilution by FACS analysis on day 2 and day 5.

Proliferation assays

Proliferation measured by thymidine incorporation: cells of interest were cultured in the presence of varying concentrations of plate bound anti-CD3. After 48 h, 1 μCi [^{3}H]thymidine (PerkinElmer Life Sciences, Foster City, CA) was added to the culture. After 12–18 hours, cells were harvested using a Harvester 96 (Tomtec, Orange, CT). Incorporated radioactivity was quantified on a 1206 Wallac Betaplate beta counter (Perkin-Elmer Life Sciences). Counts from triplicate cultures were averaged.

Immunofluorescence

Cos-7 cells (ATCC, Manassas, VA) were cultured on chamber slides (Lab-Tec II, Nunc, Inc Naperville, IL) and transfected using Fugene 6 (Roche Applied Science, Indianapolis IN). After 24 hours, cells were rinsed with PBS and fixed for 15 minutes at room temperature using 2% paraformaldehyde in PBS. Cells were permeablized by adding cold MeOH (-20° C) for 5 minutes and nonspecific antibody binding was blocked using 5% normal goat serum. The following antibodies were used to detect antigens: Myc-tag: monoclonal 9B11 (Cell Signaling, Beverly, MA), Flag-tag: monoclonal M2 (Stratagene, La Jolla, CA), PML: rabbit polyclonal H-238 (Santa Cruz Biotechnology). Primary antibodies were detected in a 2 step staining using: anti-mouse IgG1-biotin (Pharmingen #553441), anti-mouse IgG2a-biotin (Pharmingen #553388) or goat

anti-rabbit FITC (Axell), followed by a tertiary staining using streptavidin-Alexa 546 (Molecular Probes) for red images or anti-FITC Alexa 488 for green images.

Th1/Th2 differentiation assays

$CD4^+$ T cells were isolated by MACS separation (Miltenyi Biotech, Gladbach, Germany) according to the manufacturers instructions using either positive or negative selection protocols. Whole splenocytes from the same mouse that T cells were derived were irradiated (2000 rads) and used as antigen presenting cells (APC). Purified CD4 T cells were mixed with APCs at a 1:1 ratio and activated for 5 days using soluble anti-CD3ε (0.1μg/ml: 2C11, BD Pharmingen). Human IL-2 (50 U/ml) was added to all cultures and cytokines and blocking antibodies were added to cultures as follows: Th0: anti-IL4, anti-IL12 and anti-IFNγ. Th1: IL-12 and anti-IL4. Th2: IL-4 and anti-IL12 and anti-IFNγ. Cytokines and antibodies were used at the following concentrations: IL-4 50ng/ml recombinant mouse (Pierce/Endogen Rockford, IL), IL-12 40 ng/ml recombinant mouse (R&D Systems), anti-IL-4 10 μg/ml (clone 11B.11, National Cancer Institute Preclinical Repository, Rockville, MD), anti-IFNγ 10 μg/ml (clone XMG1.2, Pierce/Endogen Rockford, IL), anti-IL12 10 μg/ml (clone C17-8, Pierce/Endogen Rockford, IL) Cells were fed on day 3, or whenever medium was yellow. On day 5, cells were re-stimulated with PMA 100ng/ml and ionomycin 1 μg/ml for 5 hours. RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturers instructions, and cDNA was generated using M-MuLV reverse transcriptase (Fermentas, Hanover, MD). cDNAs were normalized by TaqMan PCR (PE Applied Biosystems, Foster City, CA) for actin using Taq polymerase (Perkin Elmer) and the following primers and probes:

Actb forward: 5'TCC TTC GTT GCC GGT CCA C3'
Actb reverse: 5'ACC AGC GCA GCG ATA TCG TC3'
Actb probe: 5'-FAM CCG CCA CCA GTT CGC CAT G-3'-Tamra

IL-4 sense 5'-AGATCATCGGCATTTTGAACG-3'
IL-4 anti-sense 5'-TTTGGCACATCCATCTCCG-3'
IL-4 probe 5'-FAM-TCACAGGAGAAGGGACGCCATGC-3'-Tamra

IL-5 sense 5'-CGCTCACCGAGCTCTGTTG-3'
IL-5 anti-sense 5'-CCAATGCATAGCTGGTGATTTTT-3'
IL-5 probe FAM-5'-CAATGAGACGATGAGGCTTCCTGTCCC-3'-Tamra

IL-13 sense 5'-GCTTATTGAGGAGCTGAGCAACA-3'
IL-13 anti-sense 5'-GGCCAGGTCCACACTCCATA-3'
IL-13 probe FAM-5'-CAAGACCAGACTCCCCTGTGCAACG-3'-Tamra

GATA-3 sense 5'-AGAACCGGCCCCTTATCAA-3'
GATA-3 anti-sense 5'-AGTTCGCGCAGGATGTCC-3'
GATA-3 probe FAM-5'-CCAAGCGAAGGCTGTCGGCAG-3'-Tamra

IFNγ sense 5'-GGATGCATTCATGAGTATTGC-3'
IFNγ anti-sense 5'-CCTTTTCCGCTTCCTGAGG-3'
IFNγ probe FAM-5'-TTTGAGGTCAACAACCCACAGGTCCA-3'-Tamra

T-bet sense 5'-CAACAACCCCTTTGCCAAAG-3'
T-bet anti-sense 5'-TCCCCCAAGCAGTTGACAGT-3'
T-bet probe FAM-5'-CCGGGAGAACTTTGAGTCCATGTACGC-3'-Tamra

Notch induced Leukemia Models

Donor mice for each experiment were selected from age and sex matched littermates that were back-crossed between 3-6 times to C57BL/6. Donor mice were injected i.p. with 5-fluorouracil (Sigma-Aldrich) at a concentration of 3 mg/20g mouse, and 4 days after treatment, the bone marrow (BM) was flushed from tibias and femurs. Red blood cells were lysed and BM cells were re-suspended at a concentration of 10^6 cells/ml in DMEM containing 15% FCS, 100 ng/ml recombinant mouse stem cell factor (R&D Systems, Minneapolis, MN), 20 ng/ml recombinant human IL-6 (R&D Systems), 10 ng/ml recombinant mouse IL-3 (R&D Systems) and incubated at 37°C. After 2 days, BM was transduced with retroviruses expressing GFP and the active intracellular domain of murine Notch1 using the MigR1 retroviral vector (a kind gift from Dr. W. Pear, University of Pennsylvania, Philadelphia, PA) and retroviral supernatants were generated using the PhoenixE packaging cell line (a protocol can be obtained from Dr. G. Nolan's web page:
(http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html).
Retroviral supernatants, supplemented with 5 μg/ml polybrene and the above

cytokines, were added to cells and spun at 2500 rpm for 120 min at 32°C. After centrifugation, cells were placed at 37°C overnight. 48 hours after retroviral transduction, BM cells were trypsinized and resuspended in serum free DMEM and 10^6 cells were injected i.v. into host mice that were lethally irradiated (1050 rads) and maintained on an antibiotic containing water. Mice were sacrificed and organs of interest were harvested after 21 to 28 days.

Lck-Notch-IC mice expressing the intracellular domain of murine Notch1 lacking the 3'-PEST domain have been described elsewhere[76].

Inhibition of Deltex4 by RNAi

Two putative RNAi sequences for Deltex4 beginning at position 562 or 1417 were cloned into the BamHI and Sal1 cloning sites of the LTRH retroviral vector[115] by generating the following oligonucleotides (underline indicates sequences derived from Deltex4)

1447-19 For: 5'-
GATCCCCGAGGATTGTACCATCTGTATTCAAGAGATACAGATGGTACAATCCTCTTTTTGGAAC

1447-19 Rev: 5'-
TCGAGTTCCAAAAAGAGGATTGTACCATCTGTATCTCTTGAATACAGATGGTACAATCCTCGGG

562-19 For: 5'-
GATCCCCGTCGGCATCACCATCCAGTTTCAAGAGAACTGGATGGTGATGCCGACTTTTTGGAAC

562-19 Rev: 5'-
TCGAGTTCCAAAAAGTCGGCATCACCATCCAGTTCTCTTGAAACTGGATGGTGATGCCGACGGG

The resulting vectors were sequenced and DNA was transfected into the PhoenixE retroviral packaging cell line to generate retroviral supernatants.

In vitro differentiation of fetal liver stem cells

Mice that were heterozygous for both Deltex1 and Deltex2 were bred together, and embryos were removed from pregnant females on day 14 to generate fetal liver. A single cell suspension was generated from each liver by pipetting gently in 1 ml of PBS containing 0.5% BSA and 2mM EDTA, and a 10μl sample was removed to isolate genomic DNA. Each liver was genotyped by PCR analysis for both Deltex1 and Deltex2 (see above) and homozygous wild type or

Deltex1/Deltex2 double knockout livers were pooled and depleted of mature lineage positive cells using DYNAL magnetic bead separation (Dynal Biotech, Brown Deer, WI) and antibodies to Ly-76 (Ter119), Mac1 (M1/70), Gr1 (8C5) and B220 (RA3-6B2). The resulting lineage negative cells were infected immediately with retroviral supernatants containing 5 μg/ml polybrene, 100 ng/ml recombinant mouse stem cell factor (R&D Systems, Minneapolis, MN) and 5 ng/ml recombinant IL-7 (PeproTech, Rocky Hill, NJ). After 48 hours cells were sorted for expression of the human CD4 marker (RPA-T4) and cKit (2B8). Sorted stem cells were plated on OP9 stromal cells and cultured in the presence of IL-7 and Flt3L as described in above.

Bibliography

1. Gerhart, J. 1998 Warkany lecture: signaling pathways in development. *Teratology* **60**, 226-39 (1999).

2. Pires-daSilva, A. & Sommer, R. J. The evolution of signalling pathways in animal development. *Nat Rev Genet* **4**, 39-49 (2003).

3. Akashi, K., Reya, T., Dalma-Weiszhausz, D. & Weissman, I. L. Lymphoid precursors. *Curr Opin Immunol* **12**, 144-50 (2000).

4. Blattman, J. N. et al. Estimating the precursor frequency of naive antigen-specific CD8 T cells. *J Exp Med* **195**, 657-64 (2002).

5. Abbas AK, L. A. a. P. J. *Cellular and Molecular Immunology* (W.B. Saunders Company, 1997).

6. Godfrey, D. I. & Zlotnik, A. Control points in early T-cell development. *Immunol Today* **14**, 547-53 (1993).

7. Jameson, S. C., Hogquist, K. A. & Bevan, M. J. Positive selection of thymocytes. *Annu Rev Immunol* **13**, 93-126 (1995).

8. Peschon, J. J. et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-60 (1994).

9. von Freeden-Jeffry, U. et al. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-26 (1995).

10. Bhandoola, A., Sambandam, A., Allman, D., Meraz, A. & Schwarz, B. Early T lineage progenitors: new insights, but old questions remain. *J Immunol* **171**, 5653-8 (2003).

11. Pear, W. S., Tu, L. & Stein, P. L. Lineage choices in the developing thymus: choosing the T and NKT pathways. *Curr Opin Immunol* **16**, 167-73 (2004).

12. Perry, S. S., Pierce, L. J., Slayton, W. B. & Spangrude, G. J. Characterization of thymic progenitors in adult mouse bone marrow. *J Immunol* **170**, 1877-86 (2003).

13. Igarashi, H., Gregory, S. C., Yokota, T., Sakaguchi, N. & Kincade, P. W. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* **17**, 117-30 (2002).

14. Gounari, F. et al. Tracing lymphopoiesis with the aid of a pTalpha-controlled reporter gene. *Nat Immunol* **3**, 489-96 (2002).

15. Godfrey, D. I., Kennedy, J., Suda, T. & Zlotnik, A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol* **150**, 4244-52 (1993).

16. Porritt, H. E. et al. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* **20**, 735-45 (2004).

17. Shortman, K. & Wu, L. Early T lymphocyte progenitors. *Annu Rev Immunol* **14**, 29-47 (1996).

18. Allman, D. et al. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* **4**, 168-74 (2003).

19. Ikawa, T., Kawamoto, H., Fujimoto, S. & Katsura, Y. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thymus revealed by a single progenitor assay. *J Exp Med* **190**, 1617-26 (1999).

20. Rothenberg, E. V. Stepwise specification of lymphocyte developmental lineages. *Curr Opin Genet Dev* **10**, 370-9 (2000).

21. von Boehmer, H. et al. Pleiotropic changes controlled by the pre-T-cell receptor. *Curr Opin Immunol* **11**, 135-42 (1999).

22. Kruisbeek, A. M. et al. Branching out to gain control: how the pre-TCR is linked to multiple functions. *Immunol Today* **21**, 637-44 (2000).

23. Hayday, A. C., Barber, D. F., Douglas, N. & Hoffman, E. S. Signals involved in gamma/delta T cell versus alpha/beta T cell lineage commitment. *Semin Immunol* **11**, 239-49 (1999).

24. MacDonald, H. R., Radtke, F. & Wilson, A. T cell fate specification and alphabeta/gammadelta lineage commitment. *Curr Opin Immunol* **13**, 219-24 (2001).

25. Kisielow, P. & von Boehmer, H. Development and selection of T cells: facts and puzzles. *Adv Immunol* **58**, 87-209 (1995).

26. Robey, E. & Fowlkes, B. J. Selective events in T cell development. *Annu Rev Immunol* **12**, 675-705 (1994).

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

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

29. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6 (1999).

30. Kojika, S. & Griffin, J. D. Notch receptors and hematopoiesis. *Exp Hematol* **29**, 1041-52 (2001).

31. Jundt, F. et al. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* **99**, 3398-403 (2002).

32. Weng, A. P. et al. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* **23**, 655-64 (2003).

33. Deftos, M. L., He, Y. W., Ojala, E. W. & Bevan, M. J. Correlating notch signaling with thymocyte maturation. *Immunity* **9**, 777-86 (1998).

34. Sade, H., Krishna, S. & Sarin, A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* **279**, 2937-44 (2004).

35. Bray, S. Notch signalling in Drosophila: three ways to use a pathway. *Semin Cell Dev Biol* **9**, 591-7 (1998).

36. Martinez Arias, A., Zecchini, V. & Brennan, K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? *Curr Opin Genet Dev* **12**, 524-33 (2002).

37. Rangarajan, A. et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *Embo J* **20**, 3427-36 (2001).

38. Mumm, J. S. & Kopan, R. Notch signaling: from the outside in. *Dev Biol* **228**, 151-65 (2000).

39. Egan, S. E., St-Pierre, B. & Leow, C. C. Notch receptors, partners and regulators: from conserved domains to powerful functions. *Curr Top Microbiol Immunol* **228**, 273-324 (1998).

40. Baron, M. et al. Multiple levels of Notch signal regulation (review). *Mol Membr Biol* **19**, 27-38 (2002).

41. Haines, N. & Irvine, K. D. Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol* **4**, 786-97 (2003).

42. Lindsell, C. E., Shawber, C. J., Boulter, J. & Weinmaster, G. Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-17 (1995).

43. Jarriault, S. et al. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol* **18**, 7423-31 (1998).

44. Shimizu, K. et al. Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol Cell Biol* **20**, 6913-22 (2000).

45. Shimizu, K., Chiba, S., Saito, T., Kumano, K. & Hirai, H. Physical interaction of Delta1, Jagged1, and Jagged2 with Notch1 and Notch3 receptors. *Biochem Biophys Res Commun* **276**, 385-9 (2000).

46. Lai, E. C. Protein degradation: four E3s for the notch pathway. *Curr Biol* **12**, R74-8 (2002).

47. Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y. & Jan, Y. N. The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc Natl Acad Sci U S A* **93**, 11925-32 (1996).

48. McGill, M. A. & McGlade, C. J. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem* **278**, 23196-203 (2003).

49. Yun, T. J. & Bevan, M. J. Notch-regulated ankyrin-repeat protein inhibits Notch1 signaling: multiple Notch1 signaling pathways involved in T cell development. *J Immunol* **170**, 5834-41 (2003).

50. Kuroda, K. et al. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity* **18**, 301-12 (2003).

51. Maillard, I., Adler, S. H. & Pear, W. S. Notch and the immune system. *Immunity* **19**, 781-91 (2003).

52. Radtke, F., Wilson, A., Mancini, S. J. & MacDonald, H. R. Notch regulation of lymphocyte development and function. *Nat Immunol* **5**, 247-53 (2004).

53. Pear, W. S. et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* **183**, 2283-91 (1996).

54. Aster, J. C. et al. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol* **20**, 7505-15 (2000).

55. Radtke, F. et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58 (1999).

56. Wilson, A., MacDonald, H. R. & Radtke, F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* **194**, 1003-12 (2001).

57. Fitzgerald, K., Harrington, A. & Leder, P. Ras pathway signals are required for notch-mediated oncogenesis. *Oncogene* **19**, 4191-8 (2000).

58. Allman, D. et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med* **194**, 99-106 (2001).

59. Wolfer, A., Wilson, A., Nemir, M., MacDonald, H. R. & Radtke, F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity* **16**, 869-79 (2002).

60. Wolfer, A. et al. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat Immunol* **2**, 235-41 (2001).

61. Huang, E. Y., Gallegos, A. M., Richards, S. M., Lehar, S. M. & Bevan, M. J. Surface expression of Notch1 on thymocytes: correlation with the double-negative to double-positive transition. *J Immunol* **171**, 2296-304 (2003).

62. Ciofani, M. et al. Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. *J Immunol* **172**, 5230-9 (2004).

63. Ciofani, M. & Zuniga-Pflucker, J. C. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol* **6**, 881-8 (2005).

64. Washburn, T. et al. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* **88**, 833-43 (1997).

65. Tanigaki, K. et al. Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* **20**, 611-22 (2004).

66. Pui, J. C. et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299-308 (1999).

67. Jaleco, A. C. et al. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med* **194**, 991-1002 (2001).

68. Schmitt, T. M. & Zuniga-Pflucker, J. C. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-56 (2002).

69. Anderson, G., Pongracz, J., Parnell, S. & Jenkinson, E. J. Notch ligand-bearing thymic epithelial cells initiate and sustain Notch signaling in thymocytes independently of T cell receptor signaling. *Eur J Immunol* **31**, 3349-54 (2001).

70. Jimenez, E. et al. Distinct mechanisms contribute to generate and change the CD4:CD8 cell ratio during thymus development: a role for the Notch ligand, Jagged1. *J Immunol* **166**, 5898-908 (2001).

71. Van Vliet, E., Jenkinson, E. J., Kingston, R., Owen, J. J. & Van Ewijk, W. Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur J Immunol* **15**, 675-81 (1985).

72. Rouse, R. V., Bolin, L. M., Bender, J. R. & Kyewski, B. A. Monoclonal antibodies reactive with subsets of mouse and human thymic epithelial cells. *J Histochem Cytochem* **36**, 1511-7 (1988).

73. Nofziger, D., Miyamoto, A., Lyons, K. M. & Weinmaster, G. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* **126**, 1689-702 (1999).

74. Doerfler, P., Shearman, M. S. & Perlmutter, R. M. Presenilin-dependent gamma-secretase activity modulates thymocyte development. *Proc Natl Acad Sci U S A* **98**, 9312-7 (2001).

75. Schmitt, T. M., Ciofani, M., Petrie, H. T. & Zuniga-Pflucker, J. C. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* **200**, 469-79 (2004).

76. Deftos, M. L., Huang, E., Ojala, E. W., Forbush, K. A. & Bevan, M. J. Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* **13**, 73-84 (2000).

77. Adler, S. H. et al. Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J Immunol* **171**, 2896-903 (2003).

78. Jiang, R. et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**, 1046-57 (1998).

79. Kumano, K. et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18**, 699-711 (2003).

80. Duncan, A. W. et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* **6**, 314-22 (2005).

81. Varnum-Finney, B. et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* **6**, 1278-81 (2000).

82. Dallman, M. J., Smith, E., Benson, R. A. & Lamb, J. R. Notch: control of lymphocyte differentiation in the periphery. *Curr Opin Immunol* **17**, 259-66 (2005).

83. Krebs, L. T., Deftos, M. L., Bevan, M. J. & Gridley, T. The Nrarp gene encodes an ankyrin-repeat protein that is transcriptionally regulated by the notch signaling pathway. *Dev Biol* **238**, 110-9 (2001).

84. Xu, T. & Artavanis-Tsakonas, S. deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. *Genetics* **126**, 665-77 (1990).

85. Gorman, M. J. & Girton, J. R. A genetic analysis of deltex and its interaction with the Notch locus in Drosophila melanogaster. *Genetics* **131**, 99-112 (1992).

86. Kishi, N. et al. Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. *Int J Dev Neurosci* **19**, 21-35 (2001).

87. Storck, S. et al. Normal immune system development in mice lacking the Deltex-1 RING finger domain. *Mol Cell Biol* **25**, 1437-45 (2005).

88. Aravind, L. The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation. *Trends Biochem Sci* **26**, 273-5 (2001).

89. Diederich, R. J., Matsuno, K., Hing, H. & Artavanis-Tsakonas, S. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120**, 473-81 (1994).

90. Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M. & Artavanis-Tsakonas, S. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121**, 2633-44 (1995).

91. Joazeiro, C. A. & Weissman, A. M. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549-52 (2000).

92. Matsuno, K. et al. Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. *Development* **129**, 1049-59 (2002).

93. Ordentlich, P. et al. Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* **18**, 2230-9 (1998).

94. Yamamoto, N. et al. Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J Biol Chem* **276**, 45031-40 (2001).

95. Izon, D. J. et al. Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity* **16**, 231-43 (2002).

96. Deftos, M. L. & Bevan, M. J. Notch signaling in T cell development. *Curr Opin Immunol* **12**, 166-72 (2000).

97. Kilby, N. J., Snaith, M. R. & Murray, J. A. Site-specific recombinases: tools for genome engineering. *Trends Genet* **9**, 413-21 (1993).

98. Ramirez-Solis, R., Davis, A. C. & Bradley, A. Gene targeting in embryonic stem cells. *Methods Enzymol* **225**, 855-78 (1993).

99. Tanigaki, K. et al. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat Immunol* **3**, 443-50 (2002).

100. Saito, T. et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* **18**, 675-85 (2003).

101. Hozumi, K. et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat Immunol* **5**, 638-44 (2004).

102. Palaga, T., Miele, L., Golde, T. E. & Osborne, B. A. TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol* **171**, 3019-24 (2003).

103. Eagar, T. N. et al. Notch 1 signaling regulates peripheral T cell activation. *Immunity* **20**, 407-15 (2004).

104. Liu, W. H. & Lai, M. Z. Deltex regulates T-cell activation by targeted degradation of active MEKK1. *Mol Cell Biol* **25**, 1367-78 (2005).

105. Lehar, S. M. & Bevan, M. J. Immunology: polarizing a T-cell response. *Nature* **430**, 150-1 (2004).

106. Maekawa, Y. et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* **19**, 549-59 (2003).

107. Minter, L. M. et al. Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat Immunol* **6**, 680-8 (2005).

108. Amsen, D. et al. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* **117**, 515-26 (2004).

109. Sacks, D. & Noben-Trauth, N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat Rev Immunol* **2**, 845-58 (2002).

110. Aster, J. C. & Pear, W. S. Notch signaling in leukemia. *Curr Opin Hematol* **8**, 237-44 (2001).

111. Bresnick, E. H., Chu, J., Christensen, H. M., Lin, B. & Norton, J. Linking Notch signaling, chromatin remodeling, and T-cell leukemogenesis. *J Cell Biochem Suppl* **Suppl 35**, 46-53 (2000).

112. Nicolas, M. et al. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* **33**, 416-21 (2003).

113. Hofmann, T. G. & Will, H. Body language: the function of PML nuclear bodies in apoptosis regulation. *Cell Death Differ* **10**, 1290-9 (2003).

114. Farley, F. W., Soriano, P., Steffen, L. S. & Dymecki, S. M. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* **28**, 106-10 (2000).

115. Barton, G. M. & Medzhitov, R. Retroviral delivery of small interfering RNA into primary cells. *Proc Natl Acad Sci U S A* **99**, 14943-5 (2002).

116. Ramain, P. et al. Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. *Curr Biol* **11**, 1729-38 (2001).

VITA

Sophie Lehar was born in England and lived in Germany until the age of 11. She moved with her family to Boston in 1975. Sophie graduated from Tufts University in 1987, with a Bachelor of Science degree in Biology and worked in several biotechnology companies including Immunogen Inc., Cambridge MA, Cambridge BioTechnology, Worcester MA and Millennium Pharmaceuticals, Cambridge MA, for 10 years before deciding to enter the Ph.D. program in Immunology at the University of Washington in Seattle.